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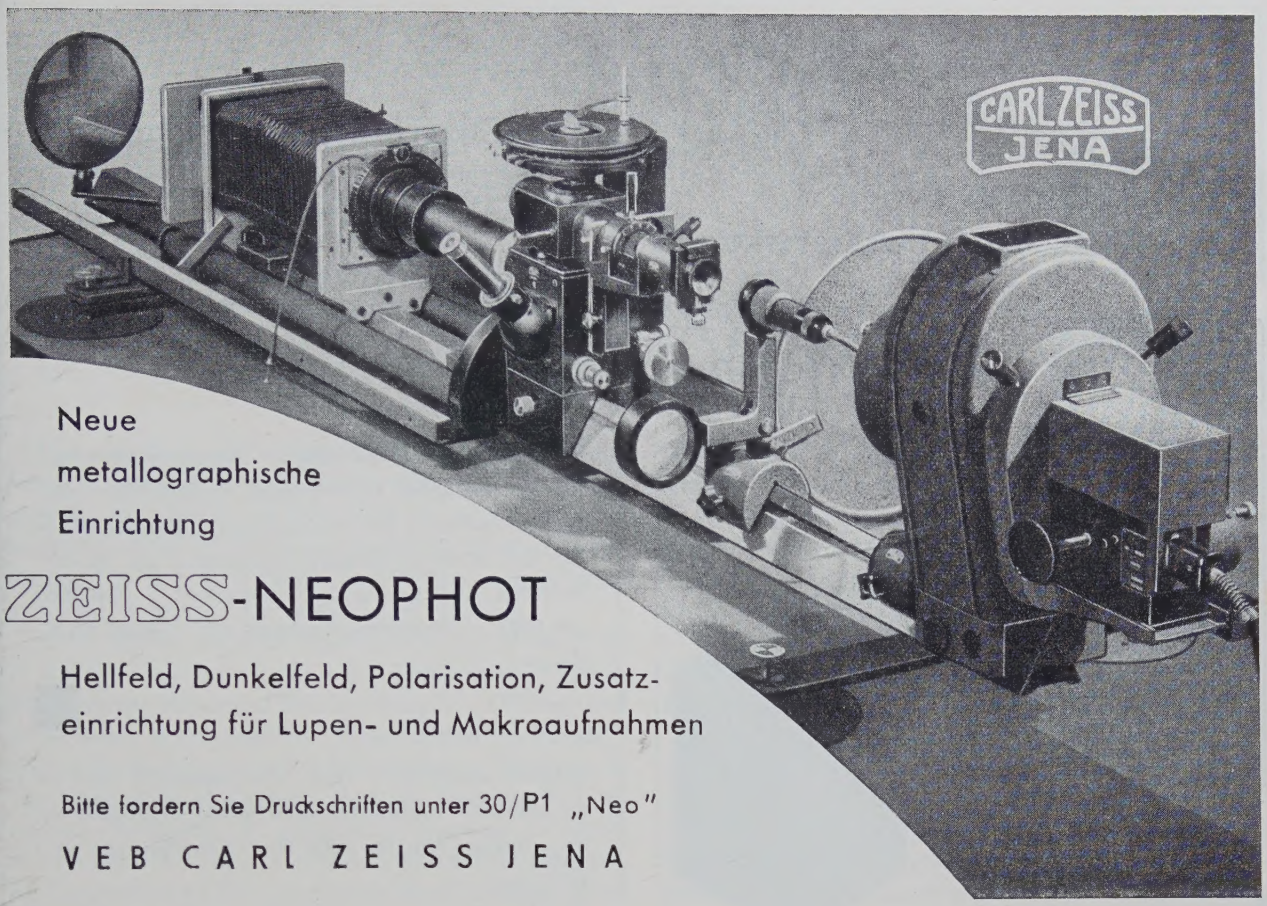

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Advances in the Field of Adrenal Cortical Hormones[1]

By A. WETTSTEIN, with the cooperation of G. ANNER, Basle[2]

The *literature* on adrenal cortical hormones has recently become so voluminous that it is no longer possible in a paper of this kind to give a detailed summary of the publications which have appeared even during the last two years. In addition, a number of excellent reviews of the subject are available.

In the purely chemical field there is that of SHOPPEE in RODDS' "Chemistry of Carbon Compounds"[3] and in the "Annual Review of Biochemistry"[4], of HEMS[5] and particularly of DJERASSI in "Vitamins and Hormones"[6] and of ROSENKRANZ and SONDHEIMER in "Progress in the Chemistry of Organic Natural Products"[7]. Concerning chemistry and metabolism there are available the reviews of SAMUELS and REICH[8], of STAUDINGER[9], of ZANDER[10], and the particularly useful monograph by DORFMAN and UNGAR[11]. Finally, the last volumes of "Recent Progress in Hormone Research"[12] and of the "Ciba Foundation Colloquia on Endocrinology"[13] are mines of compressed information. It is not attempted, therefore, to give an all-round survey on the subject, but to choose somewhat arbitrarily certain aspects of the chemistry and biochemistry of the adrenal cortical hormones and to report in more detail on aldosterone.

The adrenals are the only endocrine glands in which all groups of steroid hormones, namely cortical, progestational, androgenic and estrogenic, have been detected with certainty. Up to the present there have been isolated seven highly active *crystalline hormones* with the typical action of the adrenal cortex, namely 17-hydroxy-corticosterone (that is hydrocortisone, REICHSTEIN's substance M or KENDALL's compound F), cortisone (KENDALL's compound E), corticosterone, 11-dehydro-corticosterone (compound A of KENDALL), 17-hydroxy-cortexone (substance S of REICHSTEIN), cortexone (or 11-desoxy-corticosterone), and aldosterone (or electrocortin).

The other 23 steroids, predominantly also pregnane derivatives, which have also been obtained from the adrenals, cannot be discussed here. Their number will doubtless considerably increase as a result of the recent new investigations of adrenal extracts in connection with the isolation of aldosterone. Thus, for instance, we just obtained for the first time very considerable quantities of 11β-hydroxy-androstenedione from hog adrenals, and identified this substance chemically and physically (IR)[1]. BUSH[2] had provisionally identified this substance in the adrenal venous blood of other mammalians and SALAMON and DOBRINER[3] isolated it from the urine of patients treated with ACTH.

Occurrence. The already classical isolation work goes to the credit particularly of REICHSTEIN, of WINTERSTEINER and PFIFFNER, KENDALL and MASON, CARTLAND and KUIZENGA, of whom the last-named also worked out an especially suitable method for the preparation of adrenal extracts. Hog *adrenals* contain considerably more hormones than beef adrenals, and according to DOBRINER *et al.*[4] in descending quantities hydrocortisone, cortisone, 11-dehydro-corticosterone, substance S and corticosterone; the quantities determined by measuring the UV extinctions lie between 2 and 0·5 mg per kilo gland. Recently we have demonstrated in our own investigations[5] the presence of particularly large quantities of corticosterone, namely about 9 mg per kilo hog gland followed by 7 mg

[1] Based on a talk at the Gordon Research Conference on the Chemistry of Steroids, organized by the American Association for the Advancement of Science, New Hampton N.H., August 4, 1954. Communication No. 125 "On Steroids". No. 124 compare K. HEUSLER and A. WETTSTEIN, Chem. Ber. *87*, 1301, 1954.

[2] Research Department, Ciba Ltd., Basle.

[3] C. W. SHOPPEE and E. SHOPPEE, in ROOD, *Chemistry of Carbon Compounds*, Vol. II B (Amsterdam, 1953) p. 929.

[4] C. W. SHOPPEE, Ann. Rev. Biochem. *22*, 261 (1953).

[5] B. A. HEMS, J. Pharmacy Pharmacol. (Brit.) *5*, 409 (1953).

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[9] H. J. STAUDINGER, in L. WEISSBECKER, *Probleme des Hypophysen-Nebennierenrindensystems* (Berlin, 1953), p. 1.

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[12] G. PINCUS, Rec. Progr. Hormone Res. *7–9* (New York, 1952–1954).

[13] Ciba Foundation Colloquia on Endocrinology, 4: *Water Metabolism*, 5: *Bioassay*, 6: *Carbohydrate Metabolism*, 7: *Synthesis and Metabolism of Adrenocortical Steroids* (London, 1952–1953).

[1] R. NEHER and A. WETTSTEIN, unpublished data.

[2] I. E. BUSH, Ciba Found. Colloquia on Endocrinol. *5*, 203 (1953); *7*, 210 (1953); J. Endocrinol. *9*, 95 (1953).

[3] I. I. SALAMON and K. DOBRINER, J. Clin. Endocrinol. *12*, 967 (1952).

[4] K. DOBRINER, E. R. KATZENELLENBOGEN, and R. SCHNEIDER, Arch. Biochem. Biophys. *48*, 167 (1954).

[5] A. WETTSTEIN, F. W. KAHNT, and R. NEHER, Ciba Foundation Colloquia on Endocrinol. *8* (1954), in press; compare literature cited therein.

hydrocortisone and 3·5 mg cortisone; the latter figures fit best with those of HAINES[1], whereas the high corticosterone content is at variance with the findings of DOBRINER *et al.*[2].

As the adrenals are among the organs with the best blood supply, it is not surprising that the venous *blood* carries off larger quantities of corticosteroids per minute than corresponds to the content of the whole gland. The fairly troublesome isolation, or estimation by extraction, dialysis, chromatography, polarography in whole blood or in plasma has recently been studied intensively (for example by LEVY *et al.*[3]), NELSON and SAMUELS[4], BUSH[5] and very elegantly by MORRIS and WILLIAMS[6]. In peripheral human blood the last-named authors showed on an avarage 80γ each of hydrocortisone and corticosterone and 37γ each of 11-dehydrocorticosterone and cortisone to be present in 1 liter of plasma; these values increase 2–8 times after stimulation by ACTH. According to BUSH, corticosterone would occur only in small quantities relative to hydrocortisone. This author identified in adrenal venous blood of various kinds of animal over 74–85% of the Δ^4-3-ketones as being hydrocortisone and corticosterone. He refers especially to the characteristic ratio of these two hormones in each animal species, the human being to be classed with the sheep and monkey, and to the very high total excretion per unit body weight in the rat. Of late, interesting diurnal variations of 17α-hydroxy corticoids (e.g. hydrocortisone and cortisone in particular) have been noted in human blood[7]. The most common type has the highest content in the early morning; the level drops to about half during the forenoon, rises again somewhat towards evening, and reaches the previous maximum in the course of the night. In human *placenta*, which is known to contain large quantities of progesterone and its metabolites, two groups of workers[8] detected the presence of adrenocortical hormones by their glucocorticoid activity, or by paper chromatography. Recently the *binding* of steroids *to protein* has been investigated[9], which may give an interesting insight into the form of the hormones as circulating in the organism. Cortisone[1] and hydrocortisone[2] together with many metabolites are also excreted unchanged in the urine.

Detection, Concentration and Purification. Only a selection of the large number of publications can be briefly mentioned. The *detection*[3] is possible specially by paper chromatography and most suitably with the systems of ZAFFARONI[4] or BUSH[5] and others[6] in combination with color reactions, for example with phosphoric acid[7], or with dinitrophenylhydrazine[8]; further by utilizing the reducing properties[9], by paper electrophoresis[10], by estimation of the formaldehyde formed on oxydation[11], a method which easily gives too high values, by using the sensitivity to alkali[12], and by oxydation to 17-keto-steroids and estimation of the latter[13]; finally by comparing the specific optical rotation[14], the UV absorption, also after the action of sulfuric acid[15], and the IR spectra[16], if desired direct on

[1] W. J. HAINES, Rec. Progr. Hormone Res. *7*, 255 (1952).

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[6] C. J. O. R. MORRIS and D. C. WILLIAMS, Ciba Found. Colloquia on Endocrinol. *7*, 261 (1953).

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[2] COPE and HURLOCK, loc. cit.; For guinea pigs compare lately: S. BURSTEIN and R. I. DORFMAN, J. biol. Chem. *206*, 607 (1954); Feder .Proc. *13*, 188 (1954).

[3] Reviews see: W. J. HAINES, Rec. Progr. Hormone Res. *7*, 268 (1952). – A. ZAFFARONI, Rec. Progr. Hormone Res. *8*, 51 (1953). – K. SAVARD, Rec. Progr. Hormone Res. *9*, 185 (1954). – K. H. PFEFFER and HJ. STAUDINGER, Z. Vit. Horm. Fermentforsch. *5*, 51 (1952).

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the paper chromatogram[1]. For the *concentration* and *purification*, again partition chromatography[2], also on filter paper (see above), counter current or ordinary distribution processes, on occasion paper electrophoresis[3] and lately also dialysis[4] are used in particular.

Biological Testing. We have to limit ourselves to referring to the most recent reviews[5] which describe the very varied test methods: assay of the electrolyte metabolic activity, survival growth methods, liver glycogen methods, muscle work and stress tests, assay measuring the involution of thymus gland, eosinophil test, cytocidal action on lymphocytes, anti-inflammation tests etc. For the first isolation of aldosterone, in particular, the highly sensitive method of SIMPSON and TAIT[6] was used, determining the urinary ratio of $^{24}Na/^{42}K$, then a modified Kagawa test[7] determining Na and K (with the flame photometer) as well as the water excretion, and further the maintenance test in the adrenalectomized dog[8].

Isolation of Aldosterone. The isolation of the first 6 adrenocortical hormones in the thirties does not fall within the scope of this report. After their separation from adrenal extracts the investigators mentioned earlier found that the remaining amorphous fraction had a disproportionally strong action in survival tests using adrenalectomized animals. This action was, in fact, more pronounced than that of the most effective compound known at the time, cortexone, which was first synthesized by REICHSTEIN, but was later also detected in glandular extracts and in the blood. The crystallized hormones, were, it is true, able to explain the carbohydrate and protein activity of the adrenals; if the unlikely possibility of a synergistic effect of hormone mixtures were excluded, a hormone particularly active in the mineral metabolism was still missing. The recent isolation of this hormone should be reported here.

SIMPSON and TAIT prepared the way: with their test already mentioned, and, using the paper chromatographic method, they detected in concentrates from beef adrenals[1], then also from hog adrenals[2] and from the venous blood of the adrenals of monkeys and dogs[3], a single new substance which they called electrocortin. It was possible to differentiate this compound clearly from the similarly running cortisone[4] and to characterize it in a preliminary manner by some physico-chemical and chemical properties[5]. Of the latter the assumption that it contains an α,β-unsaturated keto group (UV) and an α-ketol (not a dioxy-acetone) side chain (negative Porter-Silber reaction[6]) was confirmed later on.

In the meantime the past-master of adrenal research, Prof. REICHSTEIN in Basle, had taken up the hunt for the new hormone, which was actively supported and supplemented by parallel investigations in Ciba. A free exchange of information took place, joint publications[7] being made with the SIMPSON and TAIT group, and the desirable mutual control of the progressive concentration was thereby guaranteed. The previously mentioned beef adrenal extracts prepared according to CARTLAND and KUIZENGA, and which were defatted in aqueous methanol by means of petroleum ether, proved to be the most valuable starting material in the beginning. The main purification consisted of a large-scale chromatography (Fig. 1) lasting more than one month, using Kieselguhr and water (1:1) as the stationary phase, and petroleum ether with increasing additions of benzene and then chloroform as the mobile phase. Adrenosterone, 11-dehydro-corticosterone and corticosterone in crystalline form were mainly obtained at first, then fractions containing cortisone and later also aldosterone and finally crystalline hydrocortisone. The fractions containing aldosterone were again purified by partition chromatography, this time in a B2 column of BUSH[8], with cellulose and aqueous methanol as the stationary and toluene-petroleum ether as the mobile phase. Some of the eluates obtained in this way from completely independent batches run in Prof. REICHSTEIN's and in our place, crystallized directly within a short interval, the presence of traces of

ELLENBOGEN, and R. N. JONES, *Infrared Absorption Spectra of Steroids* (New York, 1953).

[1] L. J. BELLAMY, J. appl. Chem. *3*, 421 (1953). – J. D. S. GOULDEN, Nature *173*, 646 (1954).

[2] J. K. N. JONES and S. R. STITCH, Biochem. J. *53*, 679 (1953). – E. R. KATZENELLENBOGEN, K. DOBRINER, and TH. H. KRITCHEVSKY, J. biol. Chem. *207*, 315 (1954).

[3] C. v. HOLT, K. D. VOIGT, and K. GAEDE, Biochem. Z. *323*, 945 (1952). – K. D. VOIGT and I. BECKMANN, Acta Endocrinol. *13*, 19 (1953). – K. D. VOIGT, W. SCHROEDER, I. BECKMANN, and H. v. D. WERTH, Acta Endocrinol. *14*, 1 (1953).

[4] L. R. AXELROD and A. ZAFFARONI, Arch. Biochem. Biophys. *50*, 347 (1954).

[5] R. I. DORFMAN, Rec. Progr. Hormone Res. *8*, 87 (1953), Physiol. Rev. *34*, 152 (1954). – D. H. NELSON, Ciba Found. Colloquia Endocrinol. *5*, 162 (1953). – D. J. INGLE, Ciba Found. Colloquia Endocrinol. *5*, 175 (1953). – M. VOGT, Ciba Found. Colloquia Endocrinol. *5*, 186 (1953). – J. A. NISSIM, Ciba Found. Colloquia Endocrinol. *5*, 193 (1953).

[6] S. A. SIMPSON and J. F. TAIT, Endocrinology *50*, 150 (1952).

[7] C. M. KAGAWA, E. G. SHIPLEY, and R. K. MEYER, Proc. Soc. exper. Biol. Med. *80*, 281 (1952). – P. DESAULLES, J. TRIPOD, and W. SCHULER, Schweiz. med. Wschr. *83*, 1088 (1953).

[8] J. J. PFIFFNER, W. W. SWINGLE, and H. M. VARS, J. biol. Chem. *104*, 701 (1934). – F. GROSS and R. MEIER, Schweiz. med. Wschr. *81*, 1013 (1951).

[1] J. F. TAIT, S. A. SIMPSON, and H. M. GRUNDY, Lancet *262*, 122 (1952).

[2] H. M. GRUNDY, S. A. SIMPSON, J. F. TAIT, and M. WOODFORD, Acta Endocrinol. *11*, 199 (1952).

[3] S. A. SIMPSON, J. F. TAIT, and I. E. BUSH, Lancet *263*, 226 (1952).

[4] H. M. GRUNDY, S. A. SIMPSON, and J. F. TAIT, Nature *169*, 795 (1952).

[5] H. M. GRUNDY, S. A. SIMPSON, J. F. TAIT, and M. WOODFORD, Acta Endocrinol. *11*, 199 (1952). – S. A. SIMPSON, and J. F. TAIT, Mem. Soc. Endocrinol. *2*, 9 (1953).

[6] C. C. PORTER and R. H. SILBER, J. biol. Chem. *185*, 201 (1950).

[7] S. A. SIMPSON, J. F. TAIT, A. WETTSTEIN, R. NEHER, J. v. EUW, and T. REICHSTEIN, Exper. *9*, 333 (1953). – S. A. SIMPSON, J. F. TAIT, A. WETTSTEIN, R. NEHER, J. v. EUW, O. SCHINDLER, and T. REICHSTEIN, Helv. chim. Acta *37*, 1163 (1954).

[8] I. E. BUSH, Biochem. J. *50*, 370 (1952); Rec. Progr. Hormone Res. *9*, 453 (1954).

moisture proving to be essential. At this point, it should be remembered that the earlier, considerably purified but very sensitive concentrates of aldosterone had defied attempts at crystallization for almost 20 years.

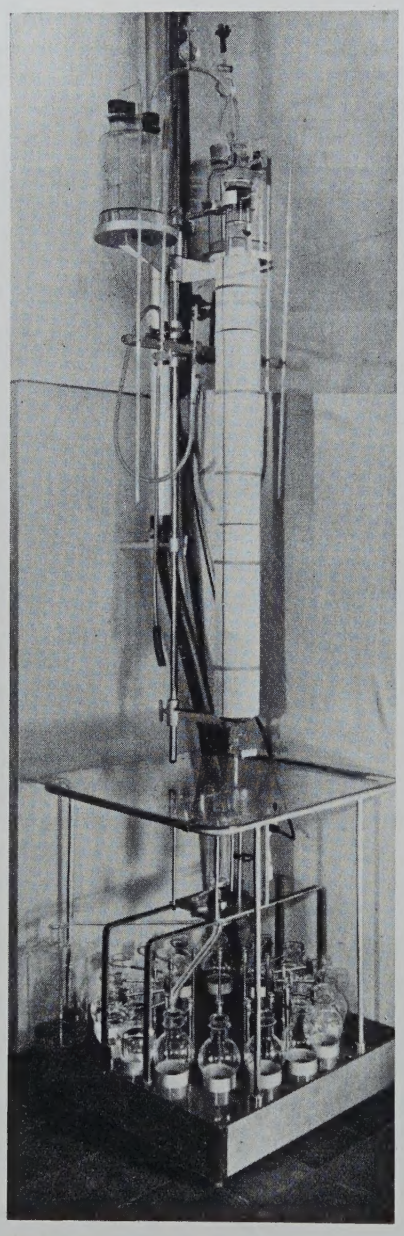
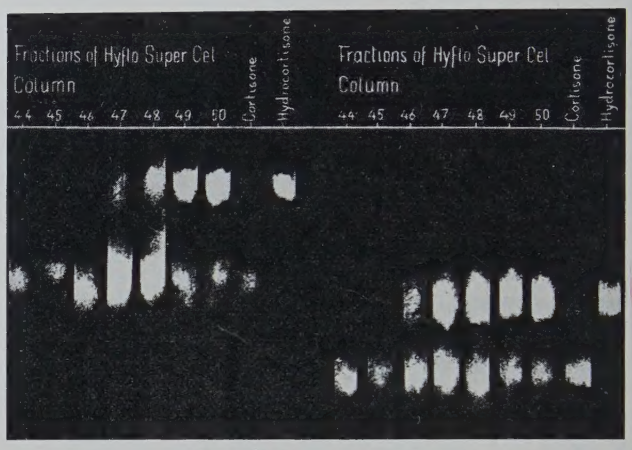


Fig. 1.

The main difficulty consisted, of course, in the ever-recurring detection of aldosterone. This was carried out, apart from biological testing, by paper chromatography (Fig. 2). In the ZAFFARONI system (propyleneglycol/toluene)[1], aldosterone travels at about the same rate, or very slightly more quickly than cortisone, but hydrocortisone considerably more slowly. On the other hand aldosterone behaves similar to hydrocortisone in BUSH's system C, whereas cortisone runs much faster[1]; in BUSH's B 5 system[2], aldosterone lies between the other two glucocorticoids. For the identification in the chromatograms the ultraviolet absorption, the power of reduction, the yellow fluorescence with sodium hydroxide and the absence of color reactions with phosphoric acid and with antimony-trichloride[3] were used in particular. About 40–95 γ aldosterone were present per kilo of fresh beef adrenals and could be largely isolated.



System: ZAFFARONI (Prop./Tol.) System: BUSH C
UV-Photocopies

Fig. 2.

As we found on an average 3·5γ aldosterone per liter of beef blood, the quantity circulating in the blood of this animal is about 70 times greater than the total content of its adrenals. In the perfusate of isolated monkey adrenal glands, aldosterone, cortisone and hydrocortisone were shown to be present in the ratio of about 1:10:1000[4] under the action of ACTH. More interesting are the values given by SIMPSON[5] for human peripheral blood without stimulation by ACTH: As she estimates the levels of hydrocortisone, determined by the H^3 and C^{14} method, at 25 γ per liter, and that of aldosterone, determined by her bioassay method, at 0·4 to 1 γ per liter, there results a ratio of aldosterone to hydrocortisone of about 1:35.

Shortly after us, MATTOX *et al.*[6] reported the successful crystallization of a strong mineralocorticoid hormone

[1] A. ZAFFARONI, R. B. BURTON, and E. H. KEUTMANN, Science *111*, 6 (1950); J. biol. Chem. *188*, 763 (1951); *193*, 749 (1951).

[1] From the differences in the area of the spots in the lower and the upper series of these two systems shown in Figure 2, it followed that in this example aldosterone was contained primarily in fractions 46–49.

[2] I. E. BUSH, Biochem. J. *50*, 370 (1952); Rec. Progr. Hormone Res. *9*, 453 (1954).

[3] R. NEHER and A. WETTSTEIN, Helv. chim. Acta *34*, 2278 (1951).

[4] H. M. GRUNDY, S. A. SIMPSON, J. F. TAIT, and M. WOODFORD, Acta Endocrinol. *11*, 199 (1952).

[5] S. A. SIMPSON and J. F. TAIT, Ciba Found. Colloqu. on Endocrinol. *8* (1954), in press.

[6] R. MATTOX, H. L. MASON, and A. ALBERT, Proc. Staff Meet. Mayo Clinic *28*, 569 (1953). – R. MATTOX, H. L. MASON, A. ALBERT, and C. F. CODE, J. Amer. chem. Soc. *75*, 4869 (1953).

from hog adrenals, by enzymatic hydrolysis of its acetate; on later direct comparison it proved to be identical with aldosterone. HAINES *et al.*[1] obtained a considerable concentration from the same starting material, FARREL and RICHARDS[2] from adrenal venous blood of dogs and MAJNARICH and DILLON[3] apparently from placental extracts. The crystallizate which VOIGT *et al.*[4] obtained from beef adrenal extracts by paper electrophoresis, amongst other methods, is doubtless, in view of its properties, different from aldosterone. LUETSCHER *et al.*[5] and also SINGER *et al.*[6] have obtained concentrates of a sodium retaining corticoid, which might be identical with aldosterone, from the urine of nephrotic and edematous patients, a particularly rich source[7]; recently it was detected biologically in the urine of normal people and of patients with cirrhosis[8]. In the case of a normal young man who received 1 mg of aldosterone by injection daily, we were able to find, by paper chromatography, about 1·5γ per 24 h in the urine; in a human adrenal tumor (CUSHING syndrome) we detected 285γ aldosterone per kilo[9].

Physiological Actions of Aldosterone. These will only be summarized here: Whereas a strict separation of the adrenocortical hormones into mineralocorticoids and glucocorticoids was more practical than correct even in earlier days (VERZÀR had already referred to the connection between potassium and carbohydrate metabolism), it has become completely untenable after the findings with crystallized aldosterone (Table I). The latter is, it is true, more than 100 times as active as cortexone[10] in SIMPSON and TAIT's test (as the earlier name "electrocortin" indicates) and possesses an extraordinary sodium retaining activity and a still somewhat greater potassium excreting activity than cortexone, but the effect on water retention is wholly lacking[1]. The permeability of muscle to the two alkali ions[2] mentioned, is strongly influenced. Aldosterone also proved correspondingly highly active in the survival test in the adrenalectomized dog[3]. Qualitative differences in action as compared with cortexone were also observed. On the other hand, however, contrary to the provisional findings of HAINES *et al.*[4], aldosterone shows some quite considerable cortisone-like actions, particularly in the liver glycogen[5], in the cold stress[6] and in the eosinophil depletion[6,7] tests, and in its ability to suppress the ACTH release[8]. Its potency here is equal to or about 1/3 of that of cortisone.

Table I.–Biological Activities of Crystalline Aldosterone (compiled by R. GAUNT[8])

Test	Potency of Aldosterone Relative to:	
	Cortexone	Cortisone
Test in which Cortexone is more potent than Cortisone		
Sodium Retention	25×	—
Potassium Excretion . . .	5×	—
Na/K Excretion	120×	—
Maintenance Adx. Dog . .	25–30×	500×
Tests in which Cortisone is more potent than Cortexone		
Cold Stress	>	= or >
Liver Glycogen	30×	1/3
Eosinophil Depletion . . .	—	1/2–1/4
ACTH Suppression	8×	1/2–1/3

Starting from her ratio of about 1:35 for the levels of aldosterone and hydrocortisone in human peripheral blood and from the potency ratio in SPEIRS' eosinophil test of 1:3, SIMPSON[9] calculates the share of gluco-

[1] R. E. KNAUFF, E. D. NIELSON, and W. J. HAINES, J. Amer. chem. Soc. *75*, 4868 (1953).

[2] G. L. FARRELL and J. B. RICHARDS, Fed. Proc. *12*, 41 (1953); Proc. Soc. exp. Biol. Med. *83*, 628 (1953); *84*, 89 (1953). According to a personal communication, Dr. FARRELL and coworkers have made it highly probable that their product is essentially homogeneous and identical with aldosterone.

[3] J. J. MAJNARICH and R. N. DILLON, Northwest Med. *50*, 677 (1951); Arch. Biochem. Biophys. *49*, 247 (1954).

[4] K. D. VOIGT and W. SCHROEDER, Naturwissenschaften *40*, 485 (1953). – W. SCHROEDER, K. D. VOIGT, and H. v. D. WERTH, Acta Endocrinol. *14*, 12 (1953).

[5] J. A. LUETSCHER, A. B. DEMING, and B. B. JOHNSON, Ciba Found. Colloquia on Endocrinol. *4*, 530 (1952). – J. A. LUETSCHER and B. B. JOHNSON, Amer. J. Med. *15*, 417 (1953); J. clin. Invest. *32*, 585 (1953); *33*, 276 (1954). Other literature is given herein. See also: J. Clin. Endocrinol. *14*, 812, 1086 (1954). Test procedure compare B. B. JOHNSON, Endocrinology *54*, 196 (1954).

[6] B. SINGER and E. H. VENNING, Endocrinology *52*, 623 (1953). – B. SINGER and J. WENER, Amer. Heart. J. *45*, 795 (1953); M. F. MCCALL and B. SINGER, J. Clin. Endocrinol. *13*, 1157 (1953).

[7] Added when reading proofs: From a concentrate of this type, kindly made available to us by Dr. LUETSCHER, we[9] were in fact able, by further chromatographic purification, to obtain considerable quantities of aldosterone in crystalline form. (Paper Exper. in press.)

[8] J. J. CHART and E. S. SHIPLEY, J. clin. Invest. *32*, 560 (1953). – C. L. COPE and J. GARCIA, Brit. med. J. *1954*, 1290. – E. S. GORDON, J. J. CHART, D. HAGEDORN, and E. G. SHIPLEY, Obstetr. and Gynecol. *4*, 39 (1954).

[9] R. NEHER and A. WETTSTEIN, Unpublished data.

[10] S. A. SIMPSON, J. F. TAIT, A. WETTSTEIN, R. NEHER, J. v. EUW, and T. REICHSTEIN, Exper. *9*, 333 (1953). – S. A. SIMPSON, J. F. TAIT, A. WETTSTEIN, R. NEHER, J. v. EUW, O. SCHINDLER, and T. REICHSTEIN, Helv. chim. Acta *37*, 1163 (1954).

[1] P. DESAULLES, J. TRIPOD, and W. SCHULER, Schweiz. med. Wschr. *83*, 1088 (1953); several papers on hormonal influences in water metabolism see Ciba Found. Colloquia on Endocrinol. *4*, 414 (1952).

[2] E. FLÜCKIGER and F. VERZÀR, Exper. *10*, 259 (1954).

[3] F. GROSS, Rec. Progr. Hormone Res. *10* (in press). – F. Gross and H. GYSEL, Acta Endocrinol. *15*, 199 (1954). – W. W. SWINGLE, R. MAXWELL, M. BEN, C. BAKER, S. J. LEBRIE, and M. EISLER, Proc. Soc. exper. Biol. Med. *86*, 147 (1954); Endocrinology *54*, 698 (1954); see also Endocrinology *54* (1954), in press.

[4] R. E. KNAUFF, E. D. NIELSON, and W. J. HAINES, J. Amer. chem. Soc. *75*, 4868 (1953).

[5] W. SCHULER, P. DESAULLES, and R. MEIER, Exper. *10*, 142 (1954).

[6] R. GAUNT, A. S. GORDON, A. A. RENZI, J. PADAWER, G. J. FRUHMAN, and M. GILMAN, J. Clin. Endocrinol. *14*, 711 (1954); Endocrinology *55*, 236 (1954).

[7] R. S. SPEIRS, S. A. SIMPSON, and J. F. TAIT, Endocrinology *55*, 233 (1954).

[8] R. GAUNT, Ciba Found. Colloquia Endocrinol. *8* (1954) (in press). – A. A. RENZI, M. GILMAN, and R. GAUNT, Proc. Soc. Exper. Biol. Med. (in press).

[9] S. A. SIMPSON and J. F. TAIT, Ciba Found. Colloqu. on Endocrinol. *8* (1954), in press.

corticoid effect of aldosterone in this blood as one hundredth of that of hydrocortisone. Assuming an opposite potency ratio of about 1000:1 for aldosterone and hydrocortisone in her labelled alkali ion test, she arrives at a ratio of 30:1 for the mineralocorticoid effect produced by the levels of these hormones in the blood.

Clinically, in ADDISON's disease, aldosterone was effective in doses of 100—200 γ per day, i.e. about 20–30 times as potent as cortexone[1]. It produced at the same time typical effects on carbohydrate metabolism and apparently caused neither an increase in blood pressure nor a pathological water retention.

Elucidation of the Constitution of Aldosterone. The noticeably broad spectrum of activity of aldosterone is readily explained by its chemical constitution. This constitution was resolved within a short time by REICHSTEIN *et al.*[2] essentially on the basis of the following findings:

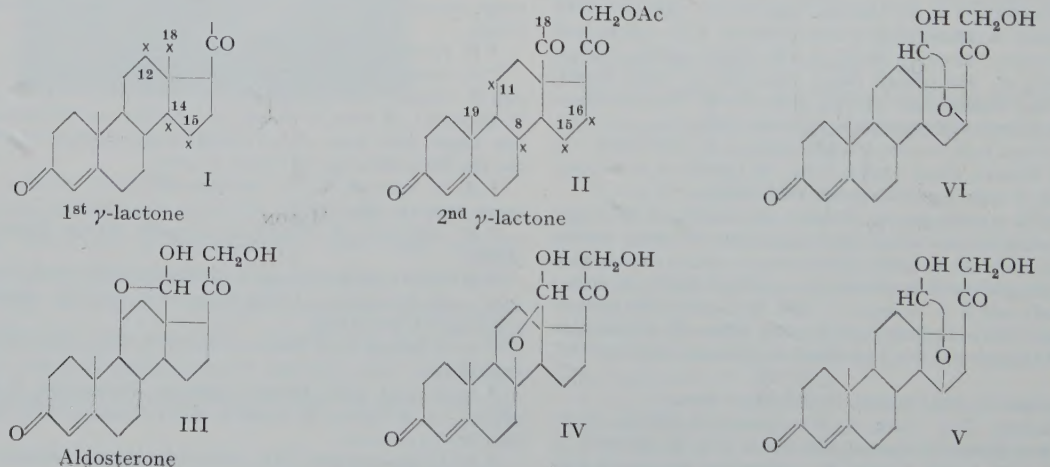
Most important, at the beginning was the microanalysis of aldosterone, one of a total of 3 determinations which it was possible to make during the entire degradation studies. It gave an empirical formula of $C_{21}H_{30}O_5 \pm H_2$, H_{28} later proving to be correct. The presence of *5* oxygen atoms has to be emphasized! On account of the ultraviolet spectrum, the reducing properties, and the formation of 1 mole formaldehyde with sodium bismuthate the working hypothesis that it is a cortexone derivative with 2 additional oxygen atoms then suggested itself. One of these must, on account of the formation of a diacetate, which in the IR no longer showed a hydroxyl group, be present as a secondary or primary hydroxyl. An indication of its position was given by degradation with $NaIO_4$, which led to a γ-lactone (Chart 1; formula I) easily detectable in the IR. This compound proved to be extraordinarily useful, as it had a very high melting point, crystallized perfectly, sublimated without decomposition in high vacuum and could thus be obtained in perfect purity even from very impure mother liquors of aldosterone. It was only this which made possible degradation in very small but yet sufficient quantity.

The hydroxyl group used in the formation of the lactone must be, we have stated, in the γ-position to the carboxyl group of the etio acid, produced by the side chain degradation, i.e. in one of the positions 12, 14, 15 or 18 marked with a cross. A 12-hydroxyl can be eliminated for steric reasons, as the 17β-oriented carboxyl group cannot react with such a hydroxyl in the presence of the also β-oriented angular 18-C atom. The 14-position can be excluded, as such a tertiary hydroxyl would have to be present in the diacetate in the free form. The choice between the 15 and 18 positions was facilitated by the fact that aldosterone-21-monoacetate again formed a γ-lactone (IR) with CrO_3, the ketol side chain with its reducing properties remaining intact. The carbonyl group of this lactone, corresponding to the original second hydroxyl, can only be in the 18 position, as all hydroxyls which are possible on the basis of the formation of the first lactone (positions 12, 14, 15, 18) lie too far from C_{19}. For the second γ-lactone, formula II thus results, with possible oxygen bridges to position 8, 11, 15 or 16 marked with a cross. As, however, aldosterone contains only 2 and not 3 free hydroxyl groups, and the first gamma lactone none at all, this oxygen ring must already have been present in aldosterone. Since a hydroxyl group has been established in position 18, this leads to one of the 4 cyclic hemiacetal structures III–VI.

[1] R. S. MACH, J. FABRE, A. DUCKERT, R. BORTH, and P. DUCOMMUN, Schweiz. med. Wschr. *84*, 407 (1954). – L. SOFFER, personal communication; G. W. THORN, Colloquium on *The Human Adrenal Cortex* II, The Ciba Foundation, London, June 1954. – A. KEKWICK and G. L. S. PAWAN, Lancet *267*, 162 (1954). – F. T. G. PRUNTY, R. R. MCSWINEY, and M. A. SMITH, Lancet *267* (1954), in press.

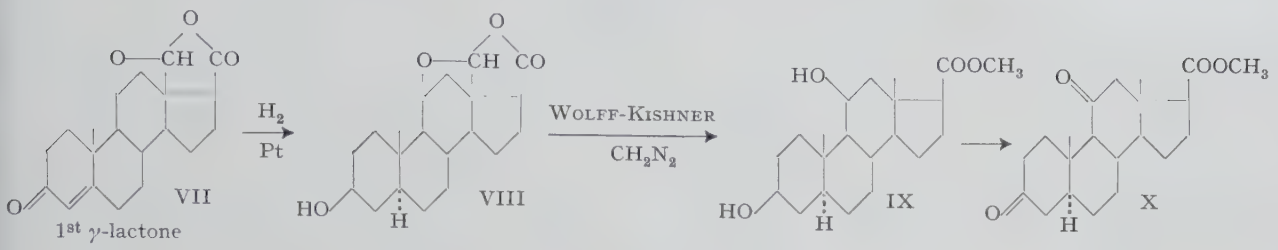
[2] S. A. SIMPSON, J. F. TAIT, A. WETTSTEIN, R. NEHER, J. v. EUW, O. SCHINDLER, and T. REICHSTEIN, Exper. *10*, 132 (1954); Helv. chim. Acta *37*, 1200 (1954).

Chart 1



Elucidation of the Constitution of Aldosterone

Chart 2



Elucidation of the Constitution of Aldosterone

Prof. Reichstein's chemical instinct instantly chose formula III for the immediate working hypothesis. The establishment of this formula and thus the proof that aldosterone is a steroid at all was made in principle in the following way (Chart 2); the first γ-lactone to which formula VII would have to be attributed, was converted, by means of hydrogenation, Wolff-Kishner reduction, methylation of the acid fraction obtained, and oxidation, into compound X, which proved to be identical with authentic methyl 3,11-diketo-etio-allocholanic acid. For steric reasons aldosterone must then be an 11β-hydroxy derivative which can be designated 18-oxo-corticosterone; in solution it is eminently present as the cyclohemiacetal of formula III, which we saw on chart 1.

This structure, which is in strong contrast to all previous speculations, readily explains the biological properties which, as stated, include most marked mineralocorticoid actions and strong glucocorticoid actions, attributable to the 11-oxygen.

Chemical Constitution and Physiological Action. Even after the elucidation of the constitution of aldosterone the fact remains that all the highly active typical hormones of the adrenal cortex represent oxygenated progesterones, i.e. cortexone (21-hydroxy-progesterone) and its further oxygenated derivatives. The additional oxygen functions enter in position 11 and/or 17, and in the case of aldosterone—for the first time—in position 18. It was, of course, of particular interest to investigate compounds representing structural or stereo-isomers of true hormones, all the more so since compounds of such a structure had been considered when discussing the possible formula of aldosterone. A group of such substances and their close relations will thus be considered before discussing new chemical methods (Chart 3).

Chart 3

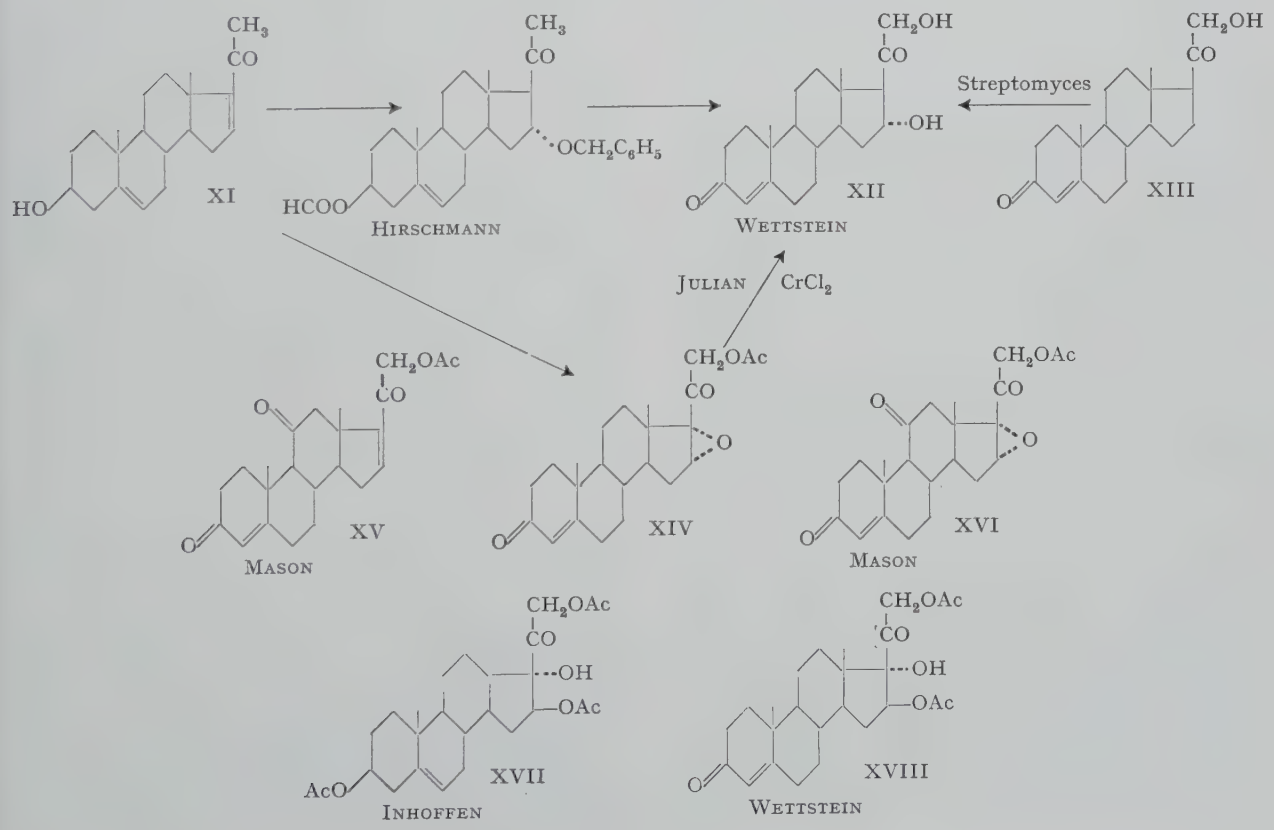
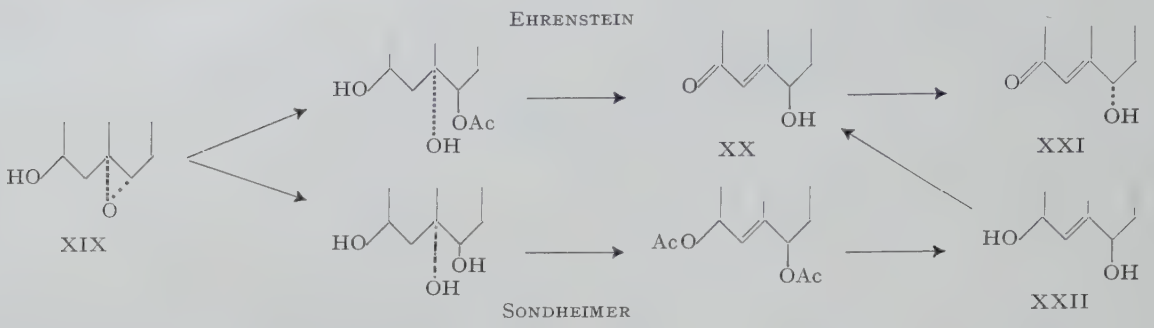


Chart 4



In this connection it was *16α-hydroxy*-cortexone (XII) which recently aroused some interest, also in view of the discovery of various 16α-hydroxylated steroid metabolites[1]. We were able to obtain it direct from cortexone (XIII) by means of the microbiological method to be discussed in detail later, involving the action of some streptomyces species[2]. Its diacetate was identical with a compound obtained by Hirschmann[3] in a chemical multistage synthesis, starting from 16-dehydro-pregnenolone (XI). The new substance XII, however, proved inactive in the glycogen and the Kagawa test and its activity in the survival test in the adrenalectomized dog was infinitesimal. The same compound was obtained simultaneously by Cole and Julian[4], also from 16-dehydro-pregnenolone (XI), via 16,17α-epoxy-cortexone acetate (XIV), using a novel reduction method with chromous salts. Speaking of *16-dehydro-* and *16,17α-epoxy* compounds, both substances of the 11-dehydro-corticosterone acetate series (XV and XVI) were found inactive as glucocorticoids and as cortisone antagonists[5]. Inhoffen[6], when synthesizing an acetylated *16β,17α-diol* of the Δ^5-21-hydroxy-pregnenolone series (XVII), also started with the 16,17α-epoxide and subjected this to acetolytic cleavage. Our findings show[7] that this compound has the constitution given by the author, while his corresponding conversion products of the pregnenolone series represent rearranged compounds of the D-Homo-series. The 16β,17α-dihydroxy-cortexone diacetate (XVIII), the 16β-acetoxy derivative of substance S now produced by us[7], proved about $^1/_{10}$ as active as free cortexone as regards sodium retention, but, in contrast to this, also caused retention of potassium, which may perhaps be the result of the relatively strong water retention observed.

Considerable work has been done on cortexones hydroxylated in position 6 (Chart 4), all the more so since, as will be discussed later, the adrenal is able to introduce a 6β-hydroxyl. This is the method by which Hayano and Dorfman[1] obtained *6β-hydroxy*-hydrocortisone, a compound which proved, however, definitely less active than hydrocortisone, and failed to show any mineralocorticoid effect. By purely chemical means 6β- and *6α-hydroxy*-cortexone (XX and XXI) were produced by Ehrenstein *et al.*[2], using acetolysis of the 5,6α-epoxide (XIX) and rearrangement of the 6β- into the 6α-compound, while Sondheimer *et al.*[3], when preparing 6β-hydroxy-cortexone, oxidized the allyl alcohol XXII with manganese dioxide. 6α-Hydroxy-cortexone caused weak sodium retention, about 4% of that of cortexone, whereas the 6β-hydroxy compound showed no detectable activity. Similar biological results have been obtained with the 6α- and 6β-hydroxy derivatives of substance S[4] (Chart 4).

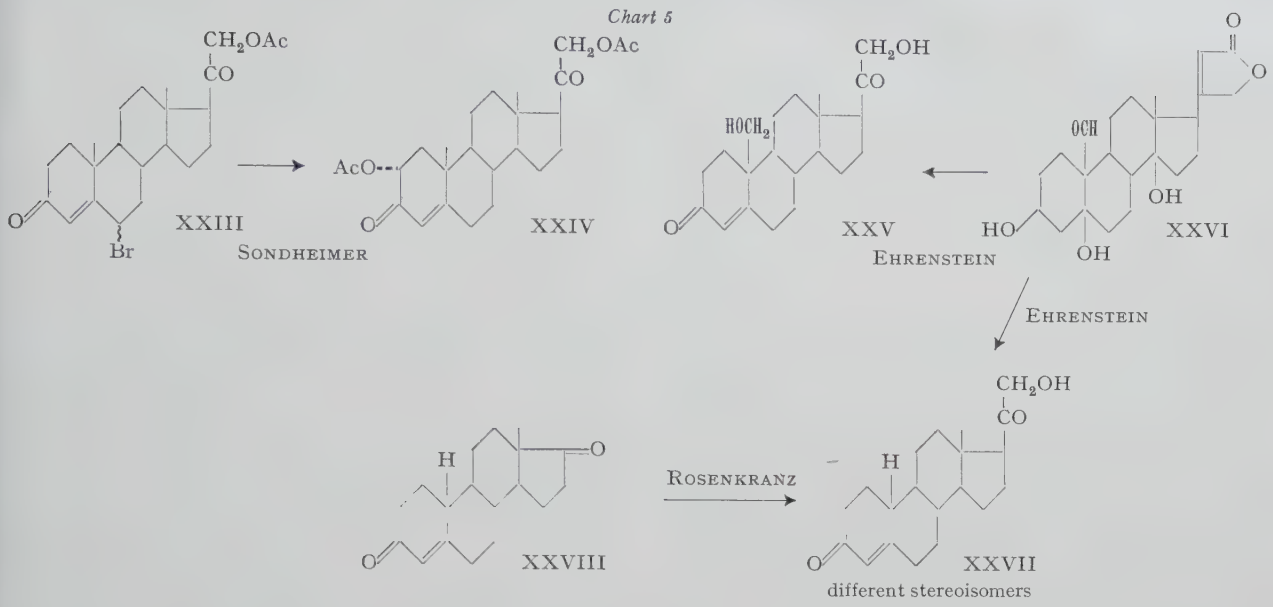
The Mexican group mentioned[5] recently identified a compound (Chart 5), obtained 15 years ago by Ehrhart *et al.* from progesterone with lead tetra-acetate, as the diacetate of *2α-hydroxy*-cortexone (XXIV). This compound could now also be obtained by rearrangement of 6-bromo-cortexone acetate (XXIII) by means of potassium acetate in glacial acetic acid. Nothing appears to have been published about the biological activity of this compound.

The *19-hydroxy*-cortexone (XXV), which is of particular interest in connection with aldosterone since it is oxygenated at the other angular methyl group—as well as the corresponding progesterone derivative—were described only recently by Barber and Ehrenstein[6]. The former produces only about

[1] See e.g. S. Liebermann, B. Praetz, Ph. Humphries, and K. Dobriner, J. biol. Chem. *204*, 491 (1953).
[2] E. Vischer, J. Schmidlin, and A. Wettstein, Helv. chim. Acta *37*, 321 (1954).
[3] H. Hirschmann, F. B. Hirschmann, and G. L. Farrell, J. Amer. chem. Soc. *75*, 4862 (1953).
[4] W. Cole and P. L. Julian, J. org. Chem. *19*, 131 (1954).
[5] W. F. McGuckin, H. L. Mason, and G. M. Higgins, Abstr. 125th Meeting Amer. chem. Soc. *1954*, 4C.
[6] H. H. Inhoffen, F. Blomeyer, and K. Brückner, Ber. dtsch. chem. Ges. *87*, 593 (1954).
[7] K. Heusler and A. Wettstein, Chem. Ber. *87*, 1301, (1954).

[1] M. Hayano and R. I. Dorfman, Arch. Biochem. Biophys. *50*, 218 (1954).
[2] P. Th. Herzig and M. Ehrenstein, J. org. Chem. *16*, 1050 (1951). – Ch. P. Balant and M. Ehrenstein, J. org. Chem. *17*, 1587 (1952).
[3] C. Amendolla, G. Rosenkranz, and F. Sondheimer, J. chem. Soc. *1954*, 1226; J. Amer. chem. Soc. *75*, 5930 (1953). – F. Sondheimer and G. Rosenkranz, Exper. *9*, 62 (1953).
[4] K. Florey and M. Ehrenstein, J. org. Chem. *19*, 1331 (1954).
[5] F. Sondheimer, St. Kaufmann, J. Romo, H. Martinez, and G. Rosenkranz, J. Amer. chem. Soc. *75*, 4712 (1953).
[6] G. W. Barber and M. Ehrenstein, J. Amer. chem. Soc. *76*, 2026 (1954).

Chart 5



4% of the sodium-retaining action of cortexone acetate and less than 2% of the glucocorticoid activity of hydrocortisone. It was obtained be means of diazoketone synthesis from a 19-acetoxy-etienic acid[1], the acid itself being available from strophanthidine (XXVI). The latter is also a possible starting material for *19-nor*-cortexone (XXVII)[2]. This cortexone derivative might have the wrong configuration in 14- and 17-position. Another 19-nor-cortexone has been obtained from estradiol after Birch-reduction according to Rosenkranz *et al.*[3] via 19-nor-androstenedione (XXVIII). This lower homologue (XXVII) of cortexone proved to be about twice as active as the parent hormone, while 19-nor-progesterone apparently is the most active gestagen known[3].

The microbiological hydroxylation in 11α-position will be considered later. All that need be said here is that 11-epicorticosterone (= *11α-hydroxy*-cortexone)[4,5] and also 11-epi-hydrocortisone[4,6] were very much less active[4] than cortexone in the survival test using epinephrectomized dogs (assuming there was any activity at all) while 11-epi-hydrocortisone was in addition without effect in the glycogen test[6]. As is known, the *12α-hydroxy* and *12-keto*-cortexone acetates produced earlier by Reichstein were also without effect. The 12α,17α-dihydroxy derivative, a hydroxyl position isomer of hydrocortisone, is said to be a cortisone antagonist[1].

The cortexone derivatives showing an additional double bond in ring C (Chart 6), and in particular the products derived from them, were recently the source of most interesting results. Some time ago we had already noted that *Δ*[11]*-anhydro*-corticosterone acetate (XXIX) resembled cortexone quantitatively in its effect in survival tests (quoted in[2]). *Δ*[9,11]*-Anhydro*-corticosterone acetate (XXX), which was first produced by Reichstein and more recently obtained by Shoppee[2], by partial synthesis from desoxycholic acid, also has an effect which resembles that of cortexone in the survival test, but is two or three times as active in the Everse-de Fremery test. Various teams of workers[3] prepared *Δ*[9,11]-anhydro-hydrocortisone acetate (XXXII) by splitting off the 11-hydroxyl group in hydrocortisone or 11-epi-hydrocortisone derivatives (XXXI).

The free alcohol of XXXII was found to be inactive in the rat liver glycogen test but quite active as an antiinflammatory by local application in the granuloma test[4]. According to Fried and Sabo[3] the acetate can serve as a suitable intermediate in the conversion of

[1] P. Th. Herzig and M. Ehrenstein, J. org. Chem. *17*, 713 (1952). – M. Ehrenstein, Chimia *6*, 287 (1952).

[2] M. Ehrenstein, J. org. Chem. *9*, 435 (1944). – G. W. Barber and M. Ehrenstein, J. org. Chem. *19*, 365 (1954).

[3] A. Sandoval, L. Miramontes, G. Rosenkranz, C. Djerassi, and F. Sondheimer, J. Amer. chem. Soc. *75*, 4117 (1953). – C. Djerassi, L. Miramontes, and G. Rosenkranz, J. Amer. chem. Soc. *75*, 4440 (1953).

[4] F. W. Kahnt, Ch. Meystre, R. Neher, E. Vischer, and A. Wettstein, Exper. *8*, 422 (1952).

[5] S. H. Eppstein, P. D. Meister, D. H. Peterson, H. C. Murray, H. M. Leigh, D. A. Lyttle, L. M. Reineke, and A. Weintraub, J. Amer. chem. Soc. *75*, 408 (1953).

[6] J. Fried, R. W. Thoma, J. R. Gerke, J. E. Herz, M. N. Donin, and D. Perlman, J. Amer. chem. Soc. *74*, 3962 (1952). – D. H. Peterson, S. H. Eppstein, P. D. Meister, B. J. Magerlein, H. C. Murray, H. M. Leigh, A. Weintraub, and L. M. Reineke, J. Amer. chem. Soc. *75*, 412 (1953).

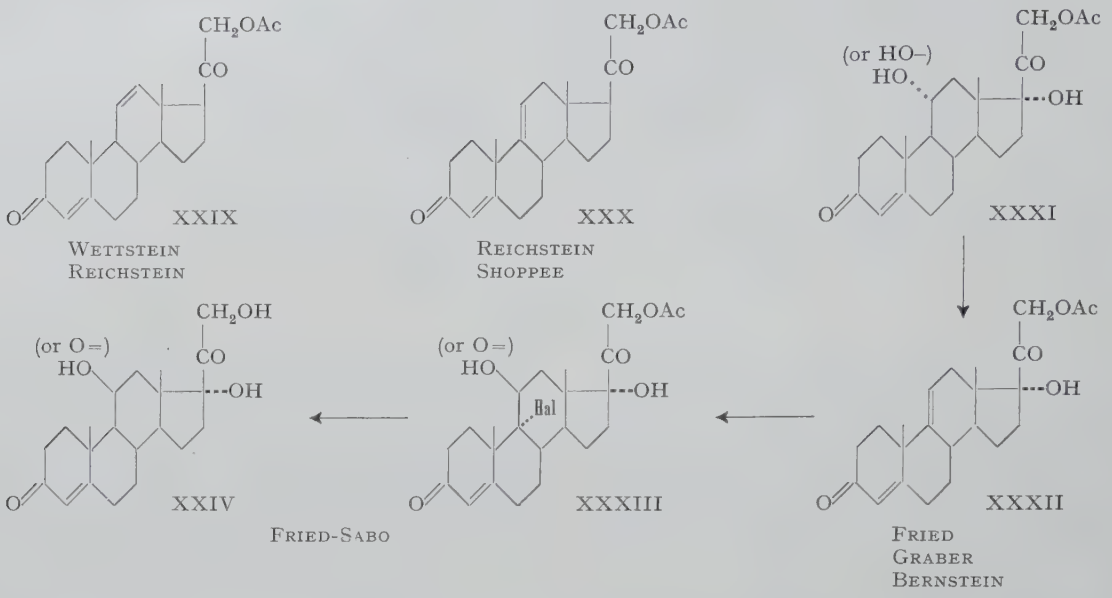
[1] W. J. Adams, B. G. Cross, A. David, F. Hartley, D. Patel, V. Petrow, and I. A. Stuart, J. Pharmacy Pharmacol. (Brit.) *5*, 861 1953).

[2] R. Casanova, C. W. Shoppee, and G. H. R. Summers, J. chem. Soc. *1953*, 2983. – R. Casanova, A. Ruff, and C. W. Shoppee, Ciba Found. Colloquia on Endocrinol. *7*, 59 (1953).

[3] J. Fried and E. F. Sabo, J. Amer. chem. Soc. *75*, 2273 (1953). – R. P. Graber, A. C. Haven, and N. L. Wendler, J. Amer. chem. Soc. *75*, 4722 (1953). – S. Bernstein, R. Littell, and J. H. Williams, J. Amer. chem. Soc. *75*, 4830 (1953).

[4] Merck Institute for Therapeutic Research. Personal communication from Dr. N. L. Wendler.

Chart 6



11-epi-hydrocortisone (XXXI) into hydrocortisone or cortisone (XXXIV); during this process it appeared that their *9α-halogenated* derivatives of formula XXXIII, which are obtained by addition of hypohalic acid to the 9,11-double bond, are yet much more interesting than the true hormones; while the latter were still superior to the acetylated 9α-iodo-hydrocortisone, 9α-bromo-cortisone and -hydrocortisone as regards glucocorticoid effect, the corresponding 9α-chloro- and in particular the 9α-fluoro-derivatives[1] proved to be 3 to 11 times more potent glucocorticoids than cortisone acetate. Moreover, the 9α-bromo and chloro derivatives in adrenalectomized rats produced a survival which was 2–20 times as long as that brought about by cortexone acetate, while the 9α-fluoro derivative was less effective than this standard substance in this respect[2]. Promising clinical results appear obtainable in addition (Thorn[3]). Partly inhibited metabolic transformation and excretion of these "unnatural" hormonal substances has been suggested as an explanation for their protracted and enhanced activity, which is found even when they are given by the oral route.

The 20-keto-*21-aldehydes* corresponding to the true hormones are also active compounds, being obtained via the nitrones, some of which are also active. While Reichstein's aldehyde of cortexone retained only a small fraction of the original activity, presumably as a result of dimerisation, Miescher's aldehyde of substance S had roughly the same activity as the basic substance. The latter is also applicable to the 21-aldehydes of cortisone and hydrocortisone[1], presumable because of the ready reducibility which was observed, e.g. with rat-liver enzymes[2], and also to the aldehyde bisulfite addition products[1]. If oxygen substitution in 21-position is dispensed with, a very considerable loss of the antiarthritic effect is observed in the case of *21-desoxy*-cortisone and -hydrocortisone[3].

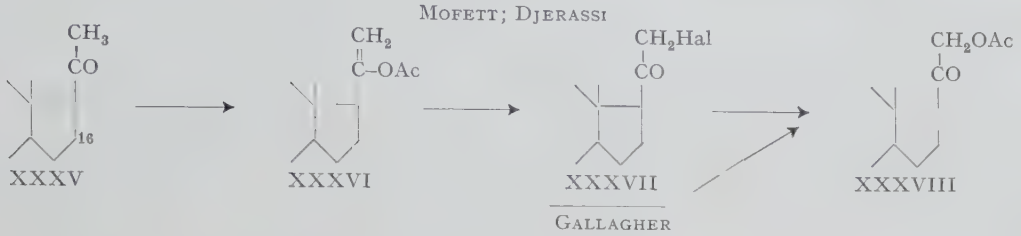
If one imagines the 4,5-double bond as being on the other side, but also in conjugation with the 3-keto group, isomeric cortisone acetates of the Δ^1-allopregnene-[4] or Δ^1-pregnene series[5], for instance, result. The first of these two still retains about 10% of the cortisone effect.

Introduction of an additional double bond, now in position 6, results in *6-dehydro*-cortisone[6], which is sometimes also found as an impurity of commercial cortisone preparations of which the double bond was introduced in the last step. Its effect upon the survival of adrenalectomized rats is similar to that of cortisone, but it brings about an additional gain in weight. In normal animals it exerts a mild lymphocytopoietic influence[7].

Enlargement of the ring D in the androstene series and construction of the side-chain resulted in the

[1] J. Fried and E. F. Sabo, J. Amer. chem. Soc. *76*, 1455 (1954).
[2] A. Borman and F. M. Singer, Fed. Proc. *13*, 185 (1954). – A. Borman, F. M. Singer, and P. Numerof, Proc. Soc. Exper. Biol. Med. *86*, 570 (1954). – See also: G. W. Liddle, M. M. Pechet, and F. C. Bartter, J. Clin. Endocrinol. *14*, 813 (1954).
[3] G. W. Thorn, Colloquium on *The Human Adrenal Cortex* II, The Ciba Foundation, London, June, 1954.
[1] E. F. Rogers, W. J. Leanza, J. P. Conbere, and K. Pfister, J. Amer. chem. Soc. *74*, 2947 (1952); *76*, 1691 (1954).
[2] J. J. Schneider, J. Amer. chem. Soc. *75*, 2024 (1953).
[3] T. H. Kritchevsky, D. L. Garmaise, and T. F. Gallagher, J. Amer. chem. Soc. *74*, 483 (1952).
[4] St. Kaufmann and J. Pataki, Exper. *7*, 260 (1951). – E. Wilson and M. Tishler, J. Amer. chem. Soc. *74*, 1609 (1952). – E. P. Oliveto, C. Gerold, and E. B. Hershberg, J. Amer. chem. Soc. *74*, 2248 (1952).
[5] V. R. Mattox and E. C. Kendall, J. biol. Chem. *188*, 287 (1951).
[6] V. R. Mattox, E. L. Woroch, G. A. Fleisher, and E. C. Kendall, J. biol. Chem. *197*, 261 (1952).
[7] G. M. Higgins, K. A. Woods, and E. C. Kendall, Endocrinology *48*, 175 (1951).

Chart 7



production of *D-homo*-cortexone acetate[1], but this possessed only about 10% of the survival effect of cortexone acetate.

A very recent synthesis was that of *17α-methyl*-cortexone acetate[2] from 17α-methyl-etio-acid. In contrast to the analogous highly active progesterone derivative, it possesses only about $^1/_5$ of the survival effect of cortexone acetate.

Methods of Partial Synthesis. The comment at the beginning of this paper about the volume of literature and of new findings in the field is particularly applicable to this chapter of adrenal research.

(a) *Side-chain degradation.* Miescher's by now already classical side-chain degradation of bile acids was recently again simplified: When working with two moles of N-bromosuccinimide in allylbromide, we[3] were able to combine into a single step three of the usual steps, i.e. α-bromination of the diphenyl-cholenes, splitting off of hydrogen bromide, and renewed bromination of the diene in position 21. The degradation of the bisnor-aldehydes, which are obtained from ergosterol or stigmasterol in particular, is suitably accomplished via their 20,22-unsaturated enol, enamine, or dehydro-nitril derivatives[4].

(b) *Ketol and dioxyacetone grouping.* The synthesis of the 20,21-ketol group according to Reichstein from the corresponding acid chlorides via the diazo-ketones is now also possible with the 11α-hydroxy- and even the 11β-hydroxy derivatives provided one protects these hydroxyl groups by converting them into the 11-formates or -trifluoroacetates, which are readily saponifiable again[5]. By this method, it was possible to synthesise, amongst others, corticosterone and 11-epi-corticosterone. Ruschig's oxalic ester method for the selective halogenation and acetoxylation of methyl-ketones in position 21 was employed by Sarett[6] in the total synthesis of cortisone, and by Ercoli[1] in the conversion of progesterone into cortexone. A new approach (Chart 7) was opened by Moffett[2] when he observed that the reaction of methyl-ketones (XXXV) with isopropenyl acetate did not produce 17,20- but 20,21-enol-acetates (XXXVI). With bromine these provide the 21-bromo-20-ketones (XXXVII) or, according to Djerassi[3], with N-iodo-succinimide, the 21-iodo-20-ketones, which are readily converted into the ketol-acetates (XXXVIII)[4]. The procedure is also applicable to 16-dehydro-20-ketones yielding 16-dehydro-20,21-ketols[5], which, according to Julian, can be converted to dihydroxy-acetone compounds via the epoxides.

The latter, one might mention, are usually still produced according to Gallagher's excellent method. This method now also permits one to obtain $\Delta^{17,20}$-monoenol-acetates from 11,20-diketones[6]. A number of teams succeeded in producing 17α-mono- and also 17,21-diacetates from dihydroxy-acetone compounds[7].

(c) *Displacement of the oxygen from position 12 to 11.* The methods of Gallagher via the 11,12-ketols (e.g. Marker-Lawson acid) and of Reichstein via the 11,12-unsaturated compounds by addition of hypohalic acid are nowadays more or less of historical interest because of their poor yields. Kendall's process via the Δ^{11}-3α,9α-oxides is of lasting technical importance in the bile-acid series and is to be prefered to the method of Heymann and Fieser, using 3β-hydroxy-3α,9α-oxido compounds, i.e. hemiketals. A novel method (Chart 8), which for the time has been described in the hecogenin series, was recently published by us[8] and

[1] R. M. Dodson, P. B. Sollman, and B. Riegel, J. Amer. chem. Soc. *75*, 5132 (1953).

[2] Ch. R. Engel and G. Just, J. Amer. chem. Soc. *76* (1954) in press. – H. Heusser, private communication.

[3] J. Heer and A. Wettstein, Helv. chim. Acta *36*, 891 (1953).

[4] M. E. Herr and F. W. Heyl, J. Amer. chem. Soc. *72*, 2617 (1950); *74*, 3627 (1952). – A. F. B. Cameron, J. S. Hunt, J. F. Oughton, P. A. Wilkinson, and B. M. Wilson, J. chem. Soc. *1953*, 3864.

[5] F. Reber, A. Lardon, and T. Reichstein, Helv. chim. Acta *37*, 45 (1954). – A. Lardon and T. Reichstein, Helv. chim. Acta *37*, 388, 443 (1954).

[6] L. H. Sarett, G. E. Arth, R. M. Lukas, R. E. Beyler, G. I. Poos, W. F. Johns, and J. M. Constantin, J. Amer. chem. Soc. *74*, 4974 (1952).

[1] A. Ercoli and P. de Ruggieri, Gazz. chim. ital. *84*, 312 (1954).

[2] R. B. Moffett and D. I. Weisblat, J. Amer. chem. Soc. *74*, 2183 (1952).

[3] C. Djerassi and C. T. Lenk, J. Amer. chem. Soc. *75*, 3493 (1953); *76*, 1722 (1954).

[4] According to Gallagher* the latter are also obtained from the 20,21-enol-acetates XXXVI with perbenzoic acid and silica gel under rearrangement. – * H. Vanderhaeghe, E. R. Katzenellenbogen, K. Dobriner, and T. F. Gallagher, J. Amer. chem. Soc. *74*, 2810 (1952). – A. H. Soloway, W. J. Considine, K. D. Fukushima, and T. F. Gallagher, J. Amer. chem. Soc. *76*, 2941 (1954).

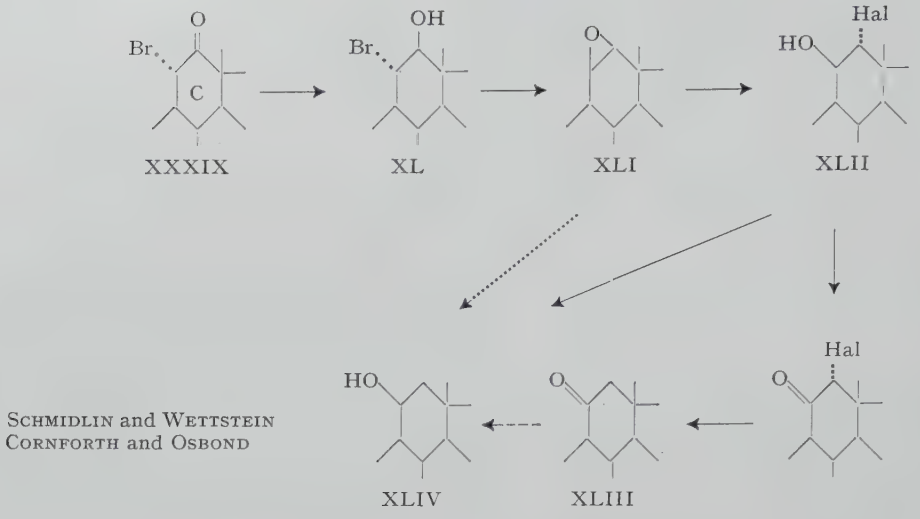
[5] Compare also: F. B. Colton, W. R. Nes, D. A. van Dorp, H. L. Mason, and E. C. Kendall, J. biol. Chem. *194*, 235 (1952). – F. B. Colton and E. C. Kendall, J. biol. Chem. *194*, 247 (1952).

[6] H. V. Anderson, E. R. Garrett, F. H. Lincoln, A. H. Nathan, and J. A. Hogg, J. Amer. chem. Soc. *76*, 743 (1954). – D. H. R. Barton, R. M. Evans, J. C. Hamlet, P. G. Jones, and T. Walker, J. Chem. Soc. *1954*, 747.

[7] R. B. Moffett and H. V. Anderson, J. Amer. chem. Soc. *76*, 747 (1954) and literature cited therein.

[8] J. Schmidlin and A. Wettstein, Helv. chim. Acta *36*, 1241 (1953).

Chart 8



Transformation of 12- into 11-Oxygenated Compounds

almost simultaneously by CORNFORTH *et al.*[1]: The 11α-bromo-12-ketone (XXXIX)[2] can be reduced with alkali-borohydrides while retaining the halogen atom, in good yield, to the 11α-bromo-12β-hydroxy compound XL. The 12β-configuration is of importance, because this compound, with alkali, or almost quantitatively with silver oxide in pyridine, now yields the 11β,12β-epoxide XLI. The latter is readily split by means of hydrohalic acids to the 11β-hydroxy-12α-halogen derivative XLII[3]. 11α,12α-Epoxides yield, as is known, only the inverse 11β-halogen-12α-hydroxy-compounds. XLII is analogous to the compounds prepared by REICHSTEIN in poor yield by the addition of hypohalic acids to 11,12-unsaturated steroids. On the one hand it can be dehydrogenated, of course, with chromium trioxide and dehalogenated with zinc to the 11-ketone XLIII, thus providing a smooth transition from 12-keto to 11-keto steroids. More interesting on the other hand is the new, direct dehalogenation of XLII, which, particularly in the case of the iodo derivative, took us almost quantitatively to the biologically important 11β-hydroxy derivative (XLIV). This is thus available from the 12-ketone in only 5 steps and in excellent yield.

(d) *The reduction of the 11-keto to the 11-hydroxy group* received very detailed attention because of the special properties of hydrocortisone as compared to cortisone. This reaction requires that other keto groups, i.e. those in position 3 and 20, are initially present in preformed form only, or that they are protected intermediately (Chart 9). WENDLER *et al.*[1] thus reduced 11-ketones with alkali-borohydrides to 11β-hydroxy derivatives, while the side-chain still contained the 17,20-dehydro-20-cyano group and the 3-keto group was protected by ketalization or semicarbazone formation (XLV). These authors were also able to produce *20*-semicarbazones from *free* 20,21-ketols (XLVI) thus achieving a protection of the 20-keto group against reduction. As is known, this is the method with which they were able for the first time to achieve a purely chemical synthesis of hydrocortisone. The protection of the 3- and also the 20-keto group by transformation into cyclic diketals (XLVII) is now frequently used; this results in Δ^5-3-alkylenedioxy derivatives from Δ^4-3-ketones, while substitution in 20-position takes place only with the free 20,21-ketols. By this means ANTONUCCI *et al.*[2] were able to convert cortisone even with the highly reactive $LiAlH_4$ into hydrocortisone plus a little 11-epi-hydrocortisone. The employment of $NaBH_4$[3] has the advantage over this that it is possible to work in the presence of water or alcohols; there is further the possibility of obtaining selective reductions of saturated, free 3,20-di- or

[1] J. W. CORNFORTH and J. M. OSBOND, Chem. and Ind. *1953*, 919. – J. W. CORNFORTH, J. M. OSBOND, and G. H. PHILIPPS, J. chem. Soc. *1954*, 907.

[2] Compare G. P. MUELLER and L. L. NORTON, J. Amer. chem. Soc. *76*, 749 (1954).

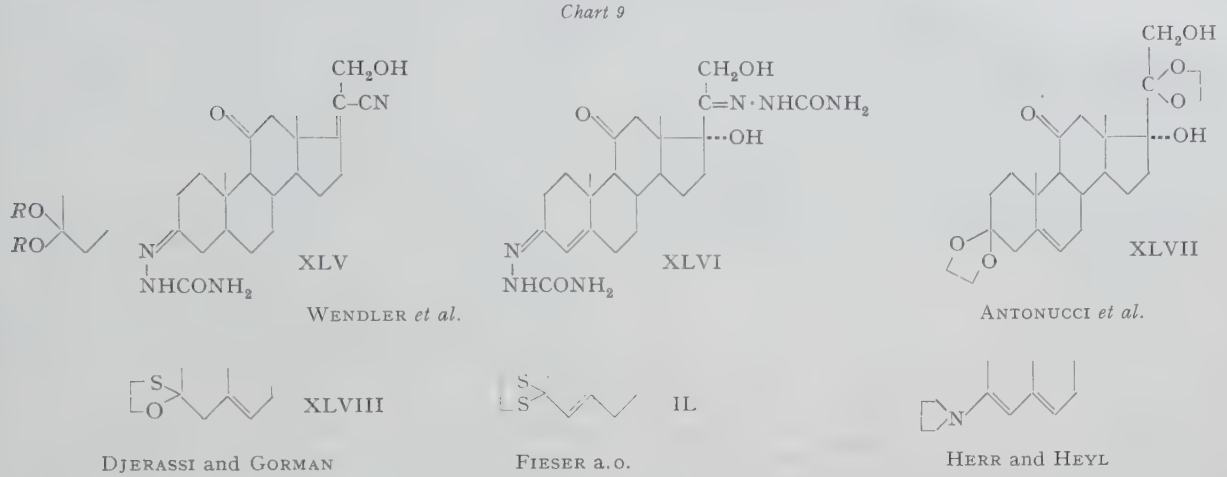
[3] Compare A. FÜRST and R. SCOTONI, Helv. chim. Acta *36*, 1410 (1953).

[1] N. L. WENDLER, R. B. GRABER, R. E. JONES, and M. TISHLER, J. Amer. chem. Soc. *72*, 5793 (1950); *74*, 3630 (1952). – N. L. WENDLER, Huang-Minlon, and M. TISHLER, J. Amer. chem. Soc. *73*, 3818 (1951).

[2] R. ANTONUCCI, S. BERNSTEIN, M. HELLER, R. LENHARD, R. LITTELL, and J. H. WILLIAMS, J. org. Chem. *18*, 70 (1953); see also R. ANTONUCCI, S. BERNSTEIN, and R. LENHARD, J. Amer. chem. Soc. *76*, 2956 (1954), furthermore R. H. LEVIN, B. J. MAGERLEIN, A. V. MCINTOSH, A. R. HANZE, G. S. FONKEN, J. L. THOMPSON, A. M. SEARCY, A. M. SCHERI, and E. S. GUTSELL, J. Amer. chem. Soc. *75*, 502 (1953); *76*, 546 (1954). – A. R. HANZE, G. S. FONKEN, A. V. MCINTOSH, A. M. SEARCY, and R. H. LEVIN, J. Amer. chem. Soc. *76*, 3179 (1954).

[3] E. P. OLIVETO, T. CLAYTON, and E. B. HERSHBERG, J. Amer. chem. Soc. *75*, 486 (1953).

Chart 9



3,11,20-triketones in position 3[1] or of Δ^4-unsaturated 3,11,20-triketones in position 20[2]. If 11α-hydroxy derivatives are desired as the main products it is expedient to reduce with sodium and propanol or with lithium, ammonia and alcohol[3].

The preparation of the ketals is best carried out, according to SARETT, by exchange dioxolanation using the ethylenedioxy derivative of mesityl oxide[4], a reaction which was recently studied in much detail by DAUBEN[5]. For certain syntheses, or for the elucidation of the constitution, cyclical alkylene hemithioketals (XLVIII)[6] or thioketals (IL)[7] should be considered, since the carbonyl functions may be regenerated therefrom with RANEY nickel alone, without the use of acids, or since they may be reduced to methylene groups.

An elegant method, also for the partial protection of carbonyl groups in polycarbonyl steroids against reduction by a metal hydride, consists in their transformation into enamines according to HERR and HEYL[8]. Pyrrolidine derivatives of Δ^4-3-ketones, e.g., are not reduced by $LiAlH_4$.

[1] O. MANCERA, A. ZAFFARONI, B. A. RUBIN, F. SONDHEIMER, G. ROSENKRANZ, and C. DJERASSI, J. Amer. chem. Soc. *74*, 3711 (1952). – A. H. SOLOWAY, A. S. DEUTSCH, and T. F. GALLAGHER, J. Amer. chem. Soc. *75*, 2356 (1953).

[2] J. K. NORYMBERSKI and G. F. WOODS, Chem. and Ind. *1954*, 518.

[3] H. HEUSSER, R. ANLIKER, and O. JEGER, Helv. chim. Acta *35*, 1537 (1952). – F. SONDHEIMER, R. YASHIN, G. ROSENKRANZ, and C. DJERASSI, J. Amer. chem. Soc. *74*, 2696 (1952). – H. L. HERZOG, E. P. OLIVETO, M. A. JEVNIK, and E. B. HERSHBERG, J. Amer. chem. Soc. *74*, 4470 (1952). – S. BERNSTEIN, R. LITTELL, and J. H. WILLIAMS, J. Amer. chem. Soc. *75*, 1481 (1953). – E. P. OLIVETO, H. L. HERZOG, and E. B. HERSHBERG, J. Amer. chem. Soc. *75*, 1505 (1953).

[4] J. M. CONSTANTIN, A. C. HAVEN, and L. H. SAERTT, J. Amer. chem. Soc. *75*, 1716 (1953).

[5] H. J. DAUBEN, B. LÖKEN, and H. J. RINGOLD, J. Amer. chem. Soc. *76*, 1359 (1954).

[6] C. DJERASSI and M. GORMAN, J. Amer. chem. Soc. *75*, 3704 (1953).

[7] L. F. FIESER, J. Amer. chem. Soc. *76*, 1945 (1954).

[8] M. E. HERR and F. W. HEYL, J. Amer. chem. Soc. *74*, 3627 (1952); *75*, 1918, 5927 (1953).

(e) *The chemical introduction of 11-oxygen into steroids with ring C unsubstituted* is fundamentally based on advancing a conjugated double bond system from ring B into C, later removing this auxiliary system once more by means of reduction (Chart 10). The most important starting materials for this are ergosterol and diosgenin, both of which permit simple sidechain degradation. The conversion of the two, or of their degradation products, into the corresponding 7,8; 9,11-dienes (L) with trans-configuration of the rings A/B (allo-series) is known. The various methods of converting these dienes into 11-ketones or 11-hydroxy compounds have been described particularly clearly in the review by ROSENKRANZ and SONDHEIMER[1] and cannot be gone into within the framework of this paper[2]. The latest publications, alone, concerning these methods would provide sufficient material for a separate review. Because of the difficulties of the eventual introduction of the 4,5-double bond into the allo-series, such conversions, according to JONES, are now carried out with 7,8; 9,11-dienes in which the original 5,6-double bond is not hydrogenated but converted into a potential double bond in the form of a 5α-hydroxyl. For this one converts the triene LI via 5,8-peroxides (formulated as LII according to FIESER) by reduction and water removal into LIII[3]. In JONES' 63rd communication[4], which appeared only recently the complete synthesis of cortisone (Chart 11) via such a 5α-acetoxy triene LIV was described.

[1] G. ROSENKRANZ and F. SONDHEIMER, Progr. Chem. Org. Natur. Prod. *10*, 274 (1953.

[2] Compare however: H. HEUSSER and O. JEGER, Ciba Found. Colloquia on Endocrinol. *7*, 46 (1953). – C. DJERASSI and G. ROSENKRANZ, Ciba Found. Colloquia on Endocrinol. *7*, 79 (1953). – F. S. SPRING *et al.*, Ciba Found. Colloquia on Endocrinol. *7*, 96 (1953). – G. ROSENKRANZ *et al.*, Rec. Progr. Hormone Res. *8*, 1 (1953).

[3] H. B. HENBEST and E. R. H. JONES, Ciba Found. Colloquia on Endocrinol. *7*, 39 (1953). – H. H. INHOFFEN and W. MENGEL, Chem. Ber. *87*, 146 (1954). – A. ZÜRCHER, H. HEUSSER, O. JEGER, and P. GEISTLICH, Helv. chim. Acta *37*, 1562 (1954).

[4] P. BLADON, H. B. HENBEST, E. R. H. JONES, B. J. LOVELL, and G. F. WOODS, J. chem. Soc. *1954*, 125, and literature cited therein.

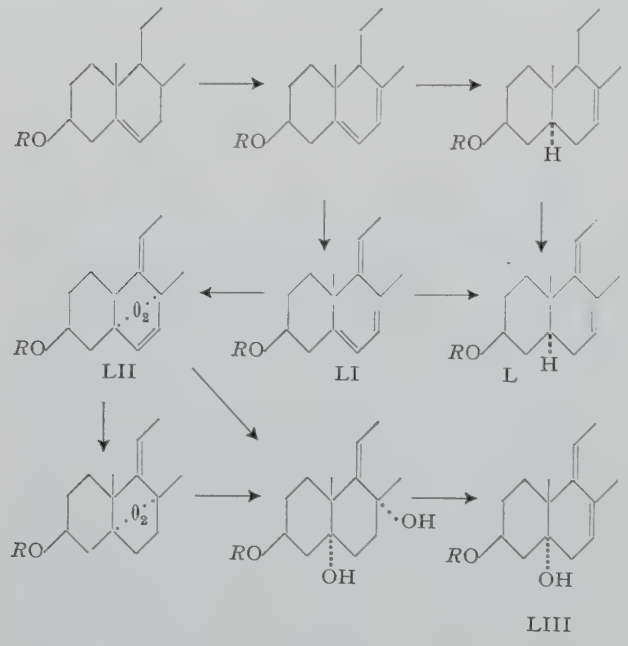
This is converted by means of what is probably the best of the currently available methods, using perbenzoic acid, then boron trifluoride, followed by acid isomerization as described by us[1] into the Δ^8-11-ketone LV, which is reduced to the saturated ketone LVI by means of lithium metal in liquid ammonia in accordance with the method of TISHLER as well as SONDHEIMER[2]. In this step the side-chain degradation to the methylketone LVII takes place, as stated earlier[1]. Since the usual process of GALLAGHER's for the introduction of the 17α-hydroxyl is too drastic for 5α-hydroxy-compounds, SARETT's method via the 17,20-dehydro nitril LVIII was chosen. Finally one produces the Δ^4-3-keto-group (LIX).

A modified route to cortisone from ergosterol under degradation of the side-chain in the 7-dehydro derivative, which takes one to the 3β-acetoxy-allopregnan-11,20-dione, was described by SPRING *et al.*[2] Unfortunately the final introduction of the Δ^4-3-keto system in this case is according to the "Standard Procedure", giving only yields of 0–10%.

A 5,8-maleic acid adduct of a 5,7;9,11-triene is said to prevent the hypertension induced by cortexone[3].

(f) The most direct method for the introduction of oxygen is the *microbiological hydroxylation*[4] (Chart 12). The starting material was chiefly progesterone (LX), which is available from cholesterol or lithocholic acid, better still from stigmasterol, and more recently again ergosterol[5], but particularly readily from sapogenins such as diosgenin. Other interesting starting materials are 17α-hydroxy-progesterone (produced according to GALLAGHER or JULIAN), cortexone (according to RUSCHIG or MOFFETT) and 17α-hydroxy-cortexone (LXIX) (REICHSTEIN's substance S; according to JULIAN). The fundamental work was done by PETERSON and MURRAY[6], who were the first to obtain from progesterone

Chart 10



[1] K. HEUSLER and A. WETTSTEIN, Helv. chim. Acta *36*, 398 (1953).

[2] E. SCHOENEWALDT, L. TURNBULL, E. M. CHAMBERLIN, D. REINHOLD, A. E. ERICKSON, W. V. RUYLE, J. M. CHEMERDA, and M. TISHLER, J. Amer. chem. Soc. *74*, 2696 (1952). – F. SONDHEIMER, R. YASHIN, G. ROSENKRANZ, and C. DJERASSI, J. Amer. chem. Soc. *74*, 2696 (1952). – Compare C. DJERASSI, W. FRICK, G. ROSENKRANZ, and F. SONDHEIMER, J. Amer. chem. Soc. *75*, 3496 (1953).

[1] M. E. HERR and F. W. HEYL, J. Amer. chem. Soc. *72*, 2617 (1950); *74*, 3627 (1952). – A. F. B. CAMERON, J. S. HUNT, J. F. OUGHTON, P. A. WILKINSON, and B. M. WILSON, J. chem. Soc. *1953*, 3864.

[2] D. MACLEAN, W. S. STRACHAN, and F. S. SPRING, Chem. and Ind. *1953*, 1259.

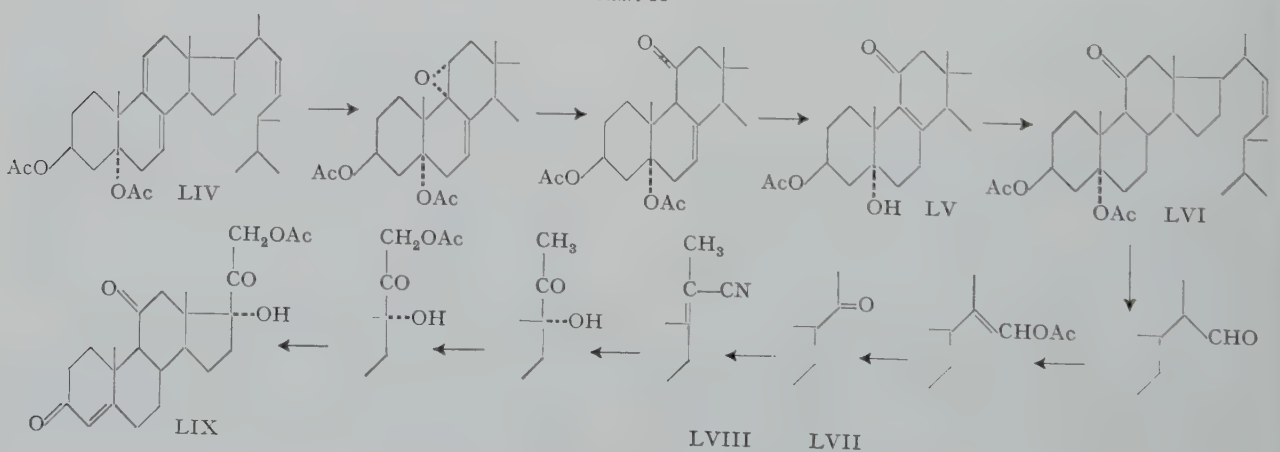
[3] R. O. STAFFORD, L. C. THOLE, and K. J. OLSON, Endocrinology *52*, 292 (1953).

[4] Reviews: D. H. PETERSON, Research *6*, 309 (1953). – K. FLOREY, Chimia *8*, 81 (1954). – R. I. DORFMAN and F. UNGAR, *Metabolism of Steroid Hormones* (Minneapolis, 1953).

[5] F. JOHNSON, G. T. NEWBOLD, and F. S. SPRING, J. chem. Soc. *1954*, 1302.

[6] D. H. PETERSON and H. C. MURRAY, J. Amer. chem. Soc. *74*, 1871 (1952).

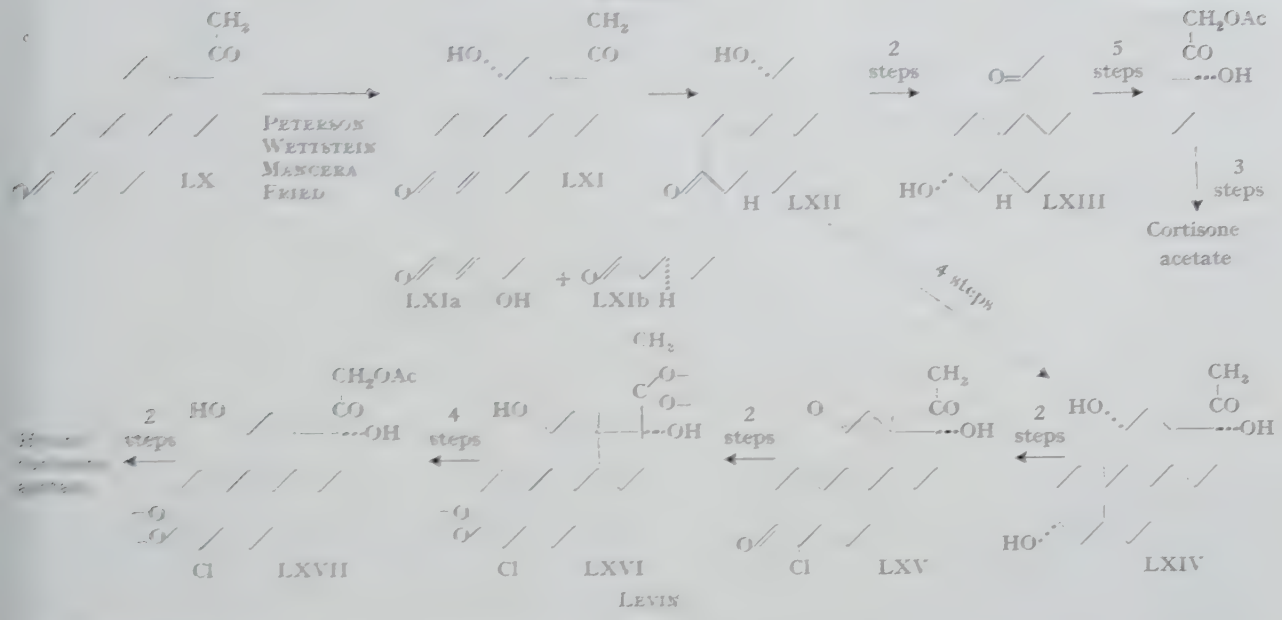
Chart 11



Synthesis of Cortisone by JONES *et al.* from Ergosterol.

Chart 12

MANCERA



LX, with rhizopus species, a 10% yield of 11α-hydroxy-progesterone (LXI). We, ourselves[1], and also MANCERA *et al.*[2], were able to increase this yield to 40–45%, while PETERSON *et al.*[3] finally raised it to about 95%; FRIED *et al.*[4] achieved a 35% yield with an aspergillus strain. In addition, and in a secondary degree, a 6β-hydroxyl enters the molecule (LXIa) or else hydrogenation of the double bond to the 5α-configuration (LXI*b*)[3] takes place. Cortexone[1,4,5], substance S (LXIX)[1,4,6], and 17α-hydroxy-progesterone[4,7], as well as 6-dehydro-progesterone[8], and pregnane or allo-pregnane-3,20-dione[9], also readily provided the 11α-, and in part once more the 6β-hydroxylation products (e.g. LXXa). In the case of substance S, simultaneous reduction of the 4-double bond to the 5β-configuration (LXX*b*)[6] occurred in addition, while with 16-dehydro-progesterone there was always a simultaneous reduction of the 16-double bond to the unnatural 17α-configuration[1]. Our findings[2] show that 3β,21-dihydroxy-allopregnane-20-one is hydroxylated in 7β-position.

Because 11α-hydroxy-progesterone (LXI) is so readily accessible, it represents a suitable intermediate in the manufacture of cortisone and hydrocortisone. MANCERA *et al.*[3] have described the preparation of the former in 9 steps which are interesting in part in view of their steric course: The catalytic hydrogenation of the double bond in the presence of the 11α-hydroxy group provides the desired 5β-derivative LXII, which yields through oxidation and partial reduction with $NaBH_4$ the 3α-hydroxy-11-ketone LXIII, familiar from the bile-acid series (3β-hydroxyl thus preferentially results in the 5α-series). Cortisone acetate is obtained therefrom by means of the usual conversion of the side-chain and introduction of the double bond.

LEVIN *et al.*[4] reached hydrocortisone in about 14 steps, starting with the intermediate LXII: they obtain LXIV by partial reduction in position 3 and introduction of the 17α-hydroxyl (according to GALLAGHER),

[1] F. W. KAHNT, CH. MEYSTRE, R. NEHER, E. VISCHER, and A. WETTSTEIN, Experientia *8*, 422 (1952). – A. WETTSTEIN, in L. WEISS-BERGER, *Probleme des Hypophysen-Nebennierenrindensystems* (Berlin, 1953), p. 23.

[2] O. MANCERA, A. ZAFFARONI, B. A. RUBIN, F. SONDHEIMER, G. ROSENKRANZ, and C. DJERASSI, J. Amer. chem. Soc. *74*, 3711 (1952).

[3] D. H. PETERSON, H. C. MURRAY, S. H. EPPSTEIN, L. M. REINEKE, A. WEINTRAUB, P. D. MEISTER, and H. M. LEIGH, J. Amer. chem. Soc. *74*, 5933 (1952).

[4] J. FRIED, R. W. THOMA, J. R. GERKE, J. E. HERZ, M. N. DONIN, and D. PERLMAN, J. Amer. chem. Soc. *74*, 3962 (1952).

[5] S. H. EPPSTEIN, P. D. MEISTER, D. H. PETERSON, H. C. MURRAY, H. M. LEIGH, D. A. LYTTLE, L. M. REINEKE, and A. WEINTRAUB, J. Amer. chem. Soc. *75*, 406 (1953).

[6] D. H. PETERSON, S. H. EPPSTEIN, P. D. MEISTER, B. J. MAGERLEIN, H. C. MURRAY, H. M. LEIGH, A. WEINTRAUB, and L. M. REINEKE, J. Amer. chem. Soc. *75*, 412 (1953).

[7] P. D. MEISTER, D. H. PETERSON, H. C. MURRAY, G. B. SPERO, S. H. EPPSTEIN, A. WEINTRAUB, L. M. REINEKE, and H. M. LEIGH, J. Amer. chem. Soc. *75*, 416 (1953).

[8] D. H. PETERSON, A. H. NATHAN, P. D. MEISTER, S. H. EPPSTEIN, H. C. MURRAY, A. WEINTRAUB, L. M. REINEKE, and H. M. LEIGH, J. Amer. chem. Soc. *75*, 419 (1953).

[9] S. H. EPPSTEIN, H. D. PETERSON, H. M. LEIGH, H. C. MURRAY, A. WEINTRAUB, L. M. REINEKE, and P. D. MEISTER, J. Amer. chem. Soc. *75*, 421 (1953).

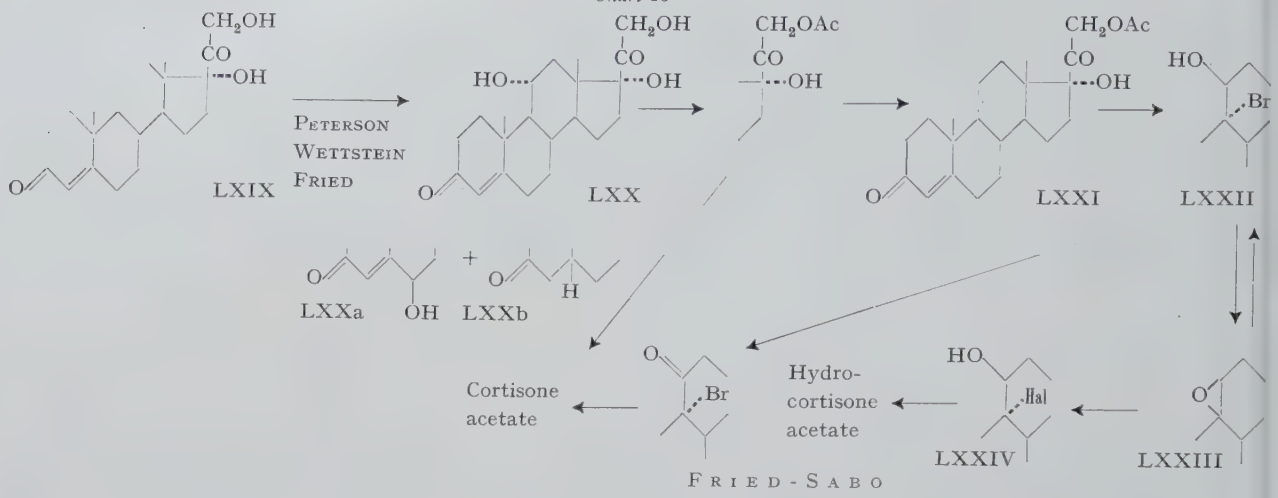
[1] P. D. MEISTER, D. H. PETERSON, H. C. MURRAY, S. H. EPPSTEIN, L. M. REINEKE, A. WEINTRAUB, and H. M. LEIGH, J. Amer. chem. Soc. *75*, 55 (1953).

[2] F. W. KAHNT, CH. MEYSTRE, R. NEHER, E. VISCHER, and A. WETTSTEIN, Experientia *8*, 422 (1952).

[3] O. MANCERA, A. ZAFFARONI, B. A. RUBIN, F. SONDHEIMER, G. ROSENKRANZ, and C. DJERASSI, J. Amer. chem. Soc. *74*, 3711 (1952). – A. H. SOLOWAY, A. S. DEUTSCH, and T. F. GALLAGHER, J. Amer. chem. Soc. *75*, 2356 (1953). – O. MANCERA, H. J. RINGOLD, C. DJERASSI, G. ROSENKRANZ, and F. SONDHEIMER, J. Amer. chem. Soc. *75*, 1286 (1953).

[4] R. H. LEVIN, B. J. MAGERLEIN, A. V. MCINTOSH, A. R. HANZE, G. S. FONKEN, J. L. THOMPSON, A. M. SEARCY, M. A. SCHERI, and E. S. GUTSELL, J. Amer. chem. Soc. *75*, 502 (1953); *76*, 546 (1954). – A. R. HANZE, G. S. FONKEN, A. V. MCINTOSH, A. M. SEARCY, and R. H. LEVIN, J. Amer. chem. Soc. *76*, 3179 (1954).

Chart 13



which is converted into LXV by coupled oxidation and chlorination by means of t-butyl hypochlorite and oxidation in position 11. The latter is hydrogenated as a diketal to the 11β-hydroxy compound LXVI, in which, after partial splitting of just the 20-ketal group, the dihydroxy-acetone side-chain is completed (LXVII). Upon finishing the Δ^4-3-keto group hydrocortisone acetate results.

It is unfortunately necessary in the two processes mentioned to saturate to the 3α-hydroxy-5β-derivative the Δ^4-3-keto group already contained in the starting material, and to reintroduce it in the end. A new, simple synthesis of hydrocortisone by FRIED and SABO[1] (Chart 13) avoids this disadvantage and also an intermediate protection of the 20-keto-group. It starts from 11-epihydrocortisone (LXX)[2], which is produced, as described, in the microbiological hydroxylation of substance S (LXIX) and comprizes 8 steps from the latter. By acetylating the 21- and esterifying the 11α-hydroxyl with p-toluenesulfonic acid, as well as splitting off the tosylate residue, one obtains 9,11-anhydro-hydrocortisone acetate (LXXI), to which is added hypobromous acid so as to obtain the 9α-bromo-11β-hydroxy derivative (LXXII). By splitting off HBr one obtains the 9β,11β-epoxide (LXXIII), which can be split up to any desired 9α-halogenated hydrocortisone acetate (LXXIV) with hydrohalic acid. The 9α-iodo derivative is particularly suitable for reduction to hydrocortisone acetate, while the 9α-chloro- and 9α-fluoro derivatives themselves represent the especially active cortical hormones mentioned earlier. The bromohydrin LXXII is converted into cortisone acetate by oxidation and debromination, but this product is, of course, also available direct by oxidizing epi-hydrocortisone-21-monoacetate. Analogous conversions were also carried out in the corticosterone series, starting with 11-epi-corticosterone.

Microorganisms, however, are able to hydroxylate steroids not only in the 11α-, but also in the 11β-position (Chart 14). HAINES *et al.*[1] were thus able to isolate hydrocortisone from the incubation of substance S with a maximum yield of about 23% when using a strain of Cunninghamella, while SHULL *et al.*[2] obtained about 40% with a Curvularia strain.

"Unnatural" microbiological hydroxylations in addition to the ones in 7β- and 11α-position were carried

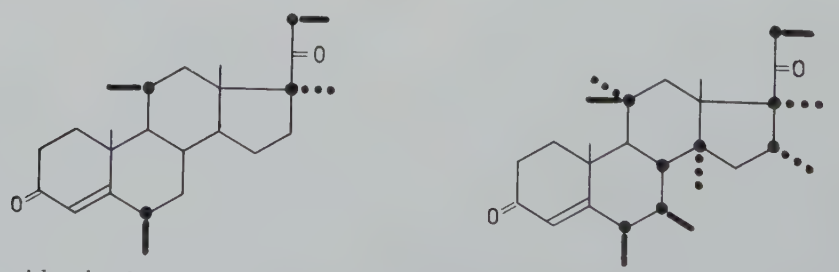
[1] J. FRIED and E. F. SABO, J. Amer. chem. Soc. *75*, 2273 (1953).

[2] Chemical syntheses see: J. ROMO, G. ROSENKRANZ, C. DJERASSI, and F. SONDHEIMER, J. Amer. chem. Soc. *75*, 1277 (1953). – E. P. OLIVETO, H. L. HERZOG, M. A. JEVNIK, H. E. JORGENSEN, and E. B. HERSHBERG, J. Amer. chem. Soc. *75*, 3651 (1953).

[1] D. R. COLINGSWORTH, J. N. KARNEMAAT, F. R. HANSON, M. P. BRUNNER, K. M. MANN, and W. J. HAINES, J. biol. Chem. *203*, 807 (1953). – F. R. HANSON, K. M. MANN, E. D. NIELSON, H. V. ANDERSON, M. P. BRUNNER, J. N. KARNEMAAT, D. R. COLINGSWORTH, and W. J. HAINES, J. Amer. chem. Soc. *75*, 5369 (1953).

[2] G. M. SHULL, R. HEIGHTS, D. A. KITA, J. HEIGHTS, and J. W. DAVISSON, US. Pat. 2658023 (1953).

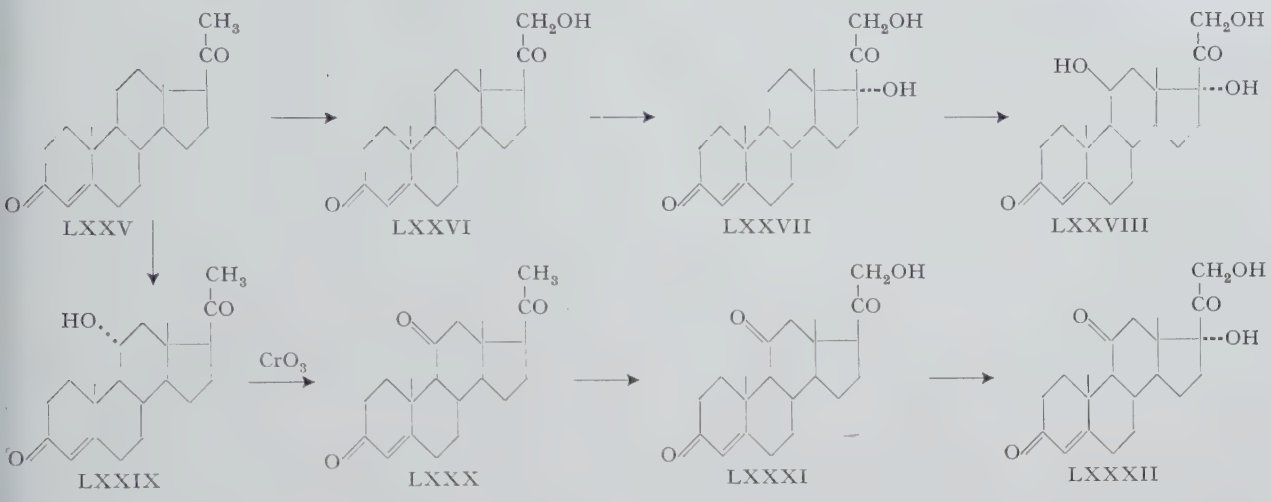
Chart 14



Adrenals: 21, 17 α, 11 β, 6 β

Microorganisms: 6 β, 7 β, 8, 11 α, 11 β, 14 α, 16 α, 17 α, 21 Hydroxylations

Chart 15



out in 8-[1], 14α-[1,2], and 16α-[3,4] position. Of the "natural" hydroxyl groups in 11β-, 17α-, and 21-position, which are essential to the action of the adrenocortical hormones, and which the adrenal gland is able to introduce in the biosynthesis, only the first has been produced up to now by microbiological means.

While investigating a considerable series of strains of fungi we[5] recently found certain organisms which are able in submerged culture to hydroxylate the C_{21}-steroids in 17α- or 21-position as well (Chart 15). The first group are Moniliaceae, in particular the Trichothecia. From Cortexone (LXXVI), for instance, they produced, apart from 6β-hydroxy-cortexone, 17α-hydroxy-cortexone (LXXVII; substance S), from 11-dehydro-corticosterone (LXXXI) they produced cortisone (LXXXII) and from corticosterone, hydrocortisone in addition to cortisone. The reaction products were generally identified by means of mixed melting points, rotation, and IR-spectra.

Hydroxylation in position 21 was achieved, in particular with Ascomycetes, such as Ophiobolus strains. Progesterone (LXXV), for instance, was thus converted into cortexone (LXXVI) with a very good yield, 11-ketoprogesterone (LXXX) into 11-dehydro-corticosterone (LXXXI), and 17α-hydroxy-progesterone into substance S. Practically no secondary products were obtained here; cortexone was left completely unchanged under the influence of such cultures.

If one combines the familiar 11-hydroxylations with these new microbiological reactions, syntheses of adrenocortical hormones can be achieved in very few steps. One path, for instance, leads from progesterone to 11α-hydroxy-progesterone and 11-keto-progesterone, which is hydroxylated in position 21 and then in position 17α, thus yielding cortisone (LXXXII) in 4 steps. Still simpler, in only 3 microbiological steps, is the conversion of progesterone into hydrocortisone (LXXVIII) via cortexone and substance S.

The new enzymatic conversions by microbiological methods appear, at least as regards the final effect, to approximate those obtained in the biosynthesis of the hormones in the adrenal. It is clear, however, that these enzyme systems of the microorganisms are less specific than those of the endocrine mammalian glands, which has advantages as regards their preparative application. Thus, for instance, the mammalian 17α-hydroxylase, which is found in particular in the adrenals and testes, is hardly able to attack 11β-hydroxylated and also 21-hydroxylated pregnane compounds[1]. This limitation does not apply to our microbiological 17α-hydroxylation.

(g) *4-unsaturated 3-keto group.* As is known, this group, in addition to the ketol group in the side-chain, is an absolute prerequisite for the adrenocortical hormone action. A more recent development, going beyond the classical method of Mattox and Kendall for the introduction of the conjugated double bond, is the elegant procedure by Levin *et al.*[2], who simultaneously oxidize the 3-hydroxyl group by means of t-butyl hypochlorite and introduce chlorine in position 4 (LXIV → LXV). During this the usually readily oxidizable 11α-hydroxyl group remains unattacked. A specific oxidation is further possible according to

[1] H. C. Murray and D. H. Peterson, US. Pat. 2602769 (1952).
[2] P. D. Meister *et al.*, Abstr. 123rd Meet. Amer. chem. Soc. *1953*, 5C.
[3] E. Vischer, J. Schmidlin, and A. Wettstein, Helv. chim. Acta *37*, 321 (1954).
[4] D. Perlman, E. Titus, and J. Fried, J. Amer. chem. Soc. *74*, 2126 (1952).
[5] Ch. Meystre, E. Vischer, and A. Wettstein, Helv. chim. Acta *37*, 1548 (1954). Added when reading the proofs: In the meantime also P. D. Meister *et al.* (J. Amer. Soc. *76*, 4050 (1954) described the microbiological introduction of the 17α-hydroxyl group.

[1] J. E. Plager and L. T. Samuels, Feder. Proc. *12*, 357 (1953); Arch. Biochem. Biophys. *42*, 477 (1953); J. biol. Chem. 1954, (in press).
[2] R. H. Levin, B. J. Magerlein, A. V. McIntosh, A. R. Hanze, G. S. Fonken, J. L. Thompson, A. M. Searcy, M. A. Scheri and E. S. Gutsell, J. Amer. chem. Soc. *75*, 502 (1953); *76*, 546 (1954). – A. R. Hanze, G. S. Fonken, A. V. McIntosh, A. M. Searcy, and R. H. Levin, J. Amer. chem Soc. *76*, 3179 (1954).

Sondheimer and Rosenkranz[1] in the case of allyl alcohols with Δ^4-3-hydroxy compounds, for instance, by the action of manganese dioxide. Mention has already been made of the again widely used method for the preparation of a Δ^4-3-keto-group from the Δ^5-3-hydroxy group via saturated 5α-hydroxy compounds (LIII)[2].

If no specifically acting oxidizing agent is used with polyhydroxy compounds, the hydroxyl groups, with the exception of the one in position 3, have to be protected intermediately. This is possible with the not readily reacting 11β-hydroxy derivatives, as previously discussed, for instance by converting into the readily resaponifiable formates[3,4] or trifluoro-acetates[3], of which even the former were able to withstand treatment with N-bromoacetamide, and were thus usable in a hydrocortisone synthesis[4]. Although it is possible to produce 11β-acetates with an acetylating agent in the presence of an acid catalyst – after the manner of the 17α-acetates earlier on[5] – and also to split partially acetate groups formed simultaneously in another position[6], the saponification of an 11β-acetate is no longer readily feasible.

Still lacking is a method, which in good yield permits the direct introduction of a double bond into 11-oxygenated, saturated 3-ketones of the 5α-(allo-)series, wherein rings A and B are in trans configuration.

Active derivatives for special purposes. In addition to the already mentioned 9α-chloro- and -fluoro-hydrocortisones and -cortisones reference should be made in particular to the relatively poorly saponifiable esters – this accounting for the protracted action – for instance the 21-trimethyl-acetate of cortexone and cortisone produced by Miescher *et al.*[7]. Desaulles and Meier[8] have thus been able to show, lately, that cortisone trimethyl-acetate in the rat has a much longer inhibiting action on the formation of the foreign body granuloma and a lower but protracted action on the wheight increase when compared with cortisone acetate of the same crystal size.

Total Synthesis. The chemically highly interesting and ever more important field of total synthesis[1] of hydrogenated steroids, and thus also of the corticoids, is too voluminous to be dealt with here, in view of the many formulae this would require. We shall therefore limit ourselves to listing the most important literature, with special reference to the names of Robinson[2], Woodward[3], Sarett[4], Johnson[5], Miescher[6], and Wilds[7].

Biosynthesis. This field, too, has developed into quite a specialty[8]. Only a certain chapter from it is therefore selected here, one to which we were recently able to make a contribution in connection with the isolation of aldosterone: *The formation of corticoids from pregnane compounds in the presence of tissue enzymes.* This work does not as yet cover the use of labeled pregnane derivatives such as progesterone, cortexone, cortisone, substance S[9] as these investigations are still in progress. Furthermore, we worked exclusively with total homogenates and not with enriched, position-specific enzyme preparations. Such were, however, used by Sweat[10] and

[1] C. Amendolla, G. Rosenkranz, and F. Sondheimer, J. chem. Soc. *1954*, 1226; J. Amer. chem. Soc. *75*, 5930 (1953). – F. Sondheimer and G. Rosenkranz, Exper. *9*, 62 (1953).

[2] H. B. Henbest and E. R. Jones, Ciba Found. Colloquia on Endocrinol. *7*, 39 (1953). – H. H. Inhoffen & W. Mengel, Chem. Ber. *87*, 146 (1954). – A. Zürcher, H. Heusser, O. Jeger & P. Geistlich, Helv. *37*, 1562 (1954). – P. Bladon, H. B. Henbest, E. R. H. Jones, B. J. Lovell, & G. F. Woods, J. Chem. Soc. *1954*, 125, and literature cited therein.

[3] F. Reber, A. Lardon, and T. Reichstein, Helv. chim. Acta *37*, 45 (1954). – A. Lardon and T. Reichstein, Helv. chim. Acta *37*, *388*, 443 (1954).

[4] E. P. Oliveto, C. Gerold, and E. B. Hershberg, Arch. Biochem. Biophys. *49*, 244 (1954).

[5] Huang-Minlon, E. Wilson, N. L. Wendler, and M. Tishler, J. Amer. chem. Soc. *74*, 5394 (1952). – R. B. Turner, J. Amer. chem. Soc. *75*, 3489 (1953).

[6] E. P. Oliveto, C. Gerold, and E. B. Hershberg, J. Amer. chem. Soc. *43*, 234 (1953). – E. P. Oliveto, C. Gerold, L. Weber, H. E. Jorgensen, R. Rausser, and E. B. Hershberg, J. Amer. chem. Soc. *75*, 5486 (1953).

[7] P. Wieland, J. Heer, J. Schmidlin, and K. Miescher, Helv. chim. Acta *34*, 354 (1951). – F. Gross and E. Tschopp, Exper. *8*, 75 (1952).

[8] P. Desaulles and R. Meier, Schweiz. med. Wschr. *84*, 741 (1954).

[1] Review see: C. A. Finch, Manufacturing Chemist *24*, 509 (1953).

[2] A. R. Pinder and R. Robinson, Nature *167*, 484 (1951). – H. M. E. Cardwell, J. W. Cornforth, S. R. Duff, H. Holtermann, and R. Robinson, Chem. and Ind. *1951*, 389; J. chem. Soc. *1953*, 361.

[3] R. B. Woodward, F. Sondheimer, D. Taub, K. Heusler, and W. M. McLamore, J. Amer. chem. Soc. *73*, 2403 (1951); *74*, 4223 (1952). – R. B. Woodward, F. Sondheimer, and D. Taub, J. Amer. chem. Soc. *73*, 3547, 3548, 4057 (1951); Ref. in Chemical Age *69*, 1222 (1953). – B. Barkley, M. W. Farrar, W. S. Knowles, and H. Raffelson, J. Amer. chem. Soc. *75*, 4110 (1953).

[4] L. H. Sarett, R. M. Lukes, G. I. Poos, J. M. Robinson, R. E. Beyler, J. M. Vandegrift, and G. E. Arth, J. Amer. chem. Soc. *74*, 1393 (1952). – R. E. Beyler and L. H. Sarett, J. Amer. chem. Soc. *74*, 1397, 1406 (1952). – R. M. Lukes, G. I. Poos and L. H. Sarett, J. Amer. chem. Soc. *74*, 1401 (1952). – L. H. Sarett, G. E. Arth, R. M. Lukes, R. E. Beyler, G. I. Poos, W. F. Johns, and J. M. Constantin, J. Amer. chem. Soc. *74*, 4974 (1952). – G. I. Poos, G. E. Arth, R. E. Beyler, and L. H. Sarett, J. Amer. chem. Soc. *75*, 422 (1953). – R. M. Lukes, G. I. Poos, R. E. Beyler, W. F. Johns, and L. H. Sarett, J. Amer. chem. Soc. *75*, 1707 (1953). – L. H. Sarett, W. F. Johns, R. E. Beyler, R. M. Lukes, G. I. Poos, and G. E. Arth, J. Amer. chem. Soc. *75*, 2112 (1953). – G. E. Arth, G. I. Poos, R. M. Lukes, F. M. Robinson, W. F. Johns, M. Feurer, and L. H. Sarett, J. Amer. chem. Soc. *76*, 1715 (1954). – R. M. Lukes and L. H. Sarett, J. Amer. chem. Soc. *76*, 1178 (1954).

[5] W. S. Johnson, B. Bannister, B. M. Bloom, A. D. Kemp, R. Pappo, E. R. Rogier, and J. Szmuszkovicz, J. Amer. chem. Soc. *75*, 2275 (1953). – W. S. Johnson, R. Pappo, and A. D. Kemp, J. Amer. chem Soc. *76*, 3353 (1954).

[6] P. Wieland, H. Überwasser, G. Anner, and K. Miescher, Helv. chim. Acta *36*, 1231 (1953).

[7] A. L. Wilds, J. W. Ralls, W. C. Wildman, and K. E. McCaleb, J. Amer. chem. Soc. *75*, 5794 (1950). – A. L. Wilds, J. W. Ralls, D. A. Tyner, R. Daniels, St. Kraychy, and M. Harnik, J. Amer. chem. Soc. *75*, 4878 (1953).

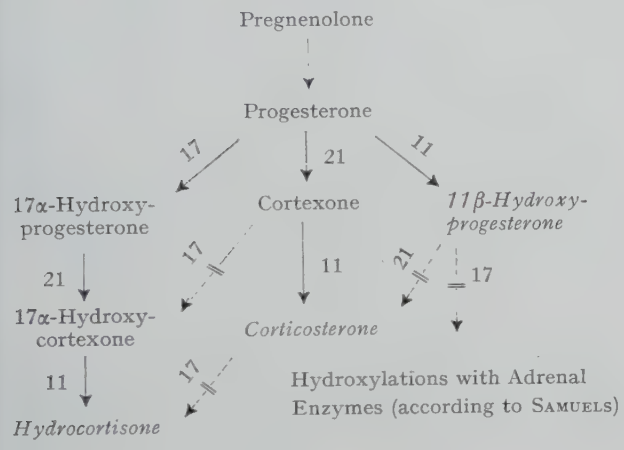
[8] Review see: O. Hechter and G. Pincus, Physiol. Rev. *34*, 459 (1954).

[9] Compare e.g.: M. Gut, Helv. chim. Acta *36*, 906 (1953). – G. I. Fujimoto and J. Prager, J. Amer. chem Soc. *75*, 3259 (1953). – L. M. Thompson, C. H. Yates, and A. D. Odell, J. Amer. chem Soc. *76*, 1194 (1954). – E. Schwenk, N. T. Werthessen and A. F. Colton, Arch. Biochem. Biophys. *48*, 322 (1954). – R. D. H. Heard *et al.*, Rec. Progr. Hormone Res. *9*, 383 (1954). – T. F. Gallagher *et al.*, Rec. Progr. Hormone Res. *9*, 411 (1954).

[10] M. L. Sweat, J. Amer. chem. Soc. *73*, 4056 (1951).

also HAYANO and DORFMAN[1] among others. These workers were able to show that enzymes in the adrenal mitochondria are responsible for the 11β-hydroxylation. As already mentioned, PLAGER and SAMUELS[2] then succeeded in demonstrating a 21-hydroxylase which proved precipitable with ammonium sulfate from the supernatant and was katalysed by ATP, and also a more unstable 17α-hydroxylase in the solution. The presence of an 11β-hydroxyl group in the reacting compound inhibited the entry of the 17- and 21-hydroxyl, while the presence of a 21-hydroxyl group prevented the entry of the 17-hydroxyl. In analogy to the earlier concepts of PINCUS-HECHTER regarding biosynthesis, SAMUELS[3] now arrives at the scheme shown in Chart 16. According to this, enzymatic hydroxylations in the series 17, 21, 11 are as yet possible only from left to right and not in the reverse order. As far as can be told this agrees with the earlier adrenal perfusion findings of the Worcester group[4].

Chart 16



Hydroxylations with Adrenal Enzymes (according to SAMUELS)

Our experiments[5] were directed in particular to obtaining the greatest possible yields of aldosterone from adrenals. They find their early origin in the work of HAINES[6], who had shown that the glucocorticoid bioactivity in hog adrenal breis can be enhanced by incubation plus aeration. Many other investigators have since worked with this method. We, ourselves[1], had been able to obtain a very considerable activation of the 11β-hydroxylation during the aerobic incubation of adrenal homogenates upon addition of substrates of the citric acid cycle, such as for instance fumarate or pyruvate, but also of nicotinamide, ATP or ascorbic acid, amongst others. This made us assume that oxidative phosphorylation is involved in the steroid 11β-hydroxylation, an assumption which has just been proved elegantly by BROWNIE and GRANT[2]. These authors show that the role of members of the citric acid cycle might be that of oxidizable substrates for oxidative phosphorylations involved in the activation of the steroids before hydroxylation.

With our previous experimental setup, it was now possible for us[3] (Table II) to increase the aldosterone content of beef adrenal homogenates, measured by paper chromatography, seven-fold by aerobic incubation in the presence of the above supplements without the addition of ATP, and as much as nineteenfold when ATP was added. The content of hydrocortisone and corticosterone even increased by 131 and 45 times respectively through incubation. Hog adrenals per se contained about 12 times as much aldosterone as beef adrenals and their content of hydrocortisone and corticosterone was 30 and 14 times higher respectively. Whereas the latter two hormones increased further by about eight times by incubation of the hog homogenates, the aldosterone content was not much affected. A clear-cut species difference becomes evident: The enzyme systems and/or precursors contained in beef adrenals, and required for the formation of the corticoids during incubation, are contained in hog adrenals to a limited degree only; on the other hand, the latter have a much higher content of *finished* corticoids. Thus in the end incubated beef adrenals, and hog adrenals with or without incubation, contain about the same quantity of aldosterone, namely approximately 1 mg per kilo.

Table II contains finally some of the preliminary experiments which concerned themselves with possible precursors in this *in-vitro* biosynthesis of aldosterone. It is evident that the addition of cortexone had an unequivocally beneficial effect; yields two or three times as great as the former optimum, and thus a maximum of more than 3 mg of aldosterone per kilo of adrenals, were demonstrable, especially in the absence of nicotinamide. Corticosterone was similarly increased, whereas hydrocortisone remained at best equal. The addition of corticosterone reduced the aldosterone value to 1/6 of the comparative experiment. Progesterone also reduced the aldosterone yield but increased

[1] M. HAYANO and R. I. DORFMAN, Feder. Proc. *11*, 228 (1952); J. biol. Chem. *200*, 175 (1953).

[2] J. E. PLAGER and L. T. SAMUELS, Feder. Proc. *11*, 383 (1952); *12*, 357 (1953); Arch. Biochem. Biophys. *42*, 477 (1953); J. biol. Chem. (1954) in press.

[3] L. T. SAMUELS, *Endocrinological Symposium*, Marburg, June 1954.

[4] O. HECHTER, Ciba Found. Colloquia on Endocrinol. *7*, 204 (1953). – Dorfman now observes a certain degree of 21-hydroxylation in 21-desoxycortisone by adrenal homogenates (Discussion subsequent to [5]).

[5] A. WETTSTEIN, F. W. KAHNT, and R. NEHER, Ciba Foundation Colloquia on Endocrinol. *8* (1954), in press; compare literature cited therein.

[6] W. J. HAINES, Rec. Progr. Hormone Res. *7*, 255 (1952).

[1] F. W. KAHNT and A. WETTSTEIN, Helv. chim. Acta *34*, 1790 (1951). – F. W. KAHNT, CH. MEYSTRE, R. NEHER, E. VISCHER, and A. WETTSTEIN, Exper. *8*, 422 (1952).

[2] A. C. BROWNIE and J. K. GRANT, Biochem. J. *57*, 255 (1954).

[3] A. WETTSTEIN, F. W. KAHNT, and R. NEHER, Ciba Foundation Colloquia on Endocrinol. *8* (1954), in press.

Table II. Aerobic Incubation of Adrenal Homogenates (according to Wettstein, Kahnt and Neher)

Adrenals	Supplements		ATP in % of Adrenals	γ Corticoids per kg Adrenals			Relative Yields		
	Nicotinamide (NA), Fumarate, Glucose			Aldosterone	Hydrocortisone	Corticosterone	Aldosterone	Hydrocortisone	Corticosterone
Beef	no incubation		—	61	230	630	1	1	1
Beef	incubated		—	425	23,500	10,600	7	100	17
Beef	incubated		0·002	764	30,200	28,500	12·5	131	45
Beef	incubated		0·2	1,145	23,900	26,300	19	104	42
Beef	incubated	Cortexone 0·05 %	0·2	2,410	22,560	80,400	40	100	128
Beef	incubated	Cortexone 0·05 %	0·2	3,360	3,760	53,000	55	16	84
Beef	(without NA) incubated	Corticosterone 0·025%	0·2	190	13,650	—	3	60	—
Beef	incubated	Progesterone 0·08 %	0·04	<475	34,400	129,000	<8	150	205
Hog	no incubation		—	765	7,080	9,050	1	1	1
Hog	incubated		0·2	1,056	58,800	64,500	1·4	8·3	7·1

that of corticosterone to an absolute maximum of about 130 mg per kilo adrenals.

In general our experiments are in agreement with the schemes drawn up by Hechter and by Samuels. They show furthermore that cortexone might be a precursor for aldosterone. In view of the results with progesterone, the 18-oxygenation should then occur after that in 21-position but, as the results with corticosterone indicate, before that in 11-position. The oxygenations leading to the formation of aldosterone would thus occur in the sequence 21-, 18-, and 11-position. This working hypothesis has to be confirmed, as said before, by tracer experiments and with purified enzymes.

Zusammenfassung

Aus den Arbeiten der letzten zwei Jahre auf dem Gebiet der Nebennierenrinden-Hormone stechen – was die Herstellung neuer, hochwirksamer Verbindungen anbetrifft – die Isolierung und Konstitutionsaufklärung des natürlichen Hormons Aldosteron und die synthetische Gewinnung der 9α-halogenierten Hydrocortisone hervor. Diese Ergebnisse haben den Weg zu weiteren wesentlichen Entwicklungen eines Gebietes geöffnet, das mit Cortison und Hydrocortison abgeschlossen schien.

Auf dem Gebiete der Teilsynthese wurde die Herstellung von 11-oxygenierten aus 12-oxygenierten Derivaten und insbesondere aus Verbindungen ohne Sauerstoff im Ring C stark ausgebaut. Bei der letzteren liegen die wichtigsten Befunde wohl in den mikrobiologischen Hydroxylierungen in 11-Stellung und den anschliessenden chemischen Umwandlungen. Erst kürzlich ist es möglich geworden, auch die typischen Seitenketten durch mikrobiologische Methoden zu gewinnen.

Die Totalsynthese der Hormone aus einfachsten chemischen Bausteinen wurde entwickelt und macht rasche Forschritte. Die Auffassungen über die Biosynthese haben konkretere Formen angenommen. Die unzähligen Arbeiten über Katabolismus und klinische Verwendung der Nebennierenrinden-Hormone konnten im Rahmen dieser Übersicht nicht einmal gestreift werden.

Brèves communications - Kurze Mitteilungen
Brevi comunicazioni - Brief Reports

Les auteurs sont seuls responsables des opinions exprimées dans ces communications. – Für die kurzen Mitteilungen ist ausschliesslich der Autor verantwortlich. – Per le brevi comunicazioni è responsabile solo l'autore. – The editors do not hold themselves responsible for the opinions expressed by their correspondents.

Intramolecular Reactions in the Oxindole Series

In his ingenious formulation of the biogenesis of the cinchona alkaloids PRELOG[1] introduced among other things a novel structural change of an indole into a quinoline nucleus;—best illustrated by the change in position of carbon atoms 1–4 from cinchonamine (I) to cinchonine (II). While this type of rearrangement is without analogy in the chemistry of indoles, it is known with compounds of higher oxidation state, as exemplified by the transformation of 3-carboxymethyloxindole (IV) into 2-hydroxy-3,4-dihydrocinchoninic acid (V). This may be of biogenetic significance especially since quinamine (III), the only other indole-containing cinchona alkaloid, also appears in a more oxidized form.

Much confusion has existed in the literature regarding the formation and stability of "oxindole-3-acetic acid" (IV) and its derivatives[2]. In fact, most structures containing the oxindole nucleus had to be revised to derivatives of 2-quinolone. Only most recently has authentic 3-carboxymethyloxindole (IV) been isolated[3]. Since all former modes of preparation of such oxindole derivatives included a hydrolytic treatment and since the six-membered lactam of o-aminophenylsuccinic acid, the hydrolysis product, is known to be more thermodynamically stable than the 5-membered cyclic amide[1,2] the rearrangement has been believed to be a consequence of a hydrolysis of the oxindole nucleus to a dibasic acid and a subsequent lactamization of the latter to a quinolone. This explanation, however, is incomplete as it ignores the relative stability of the oxindole nucleus toward hydrolysis. It thus becomes obvious that the presence of the side-chain carboxyl group is the major factor that contributes to the ease of the rearrangement. Interaction between this function and the carbonyl group of the lactam, a process well-known for γ-ketoacids, could be envisaged to yield the pseudo-acid IVa. Since the latter is also a pseudo-anhyride, it would be in equilibrium with the actual amino-anhydride which, in turn, by a similar path would form the quinolone V (as formally pictured above). The over-all equilibrium would be acid-or base-catalyzed and any of the reaction intermediates might indeed be hydrolyzed to o-aminophenylsuccinic acid under correct experimental conditions although the latter is not an essential precursor of the quinolone.

[1] R. GOUTAREL, M.-M. JANOT, V. PRELOG, and W. I. TAYLOR, Helv. chim. Acta *33*, 150 (1950).

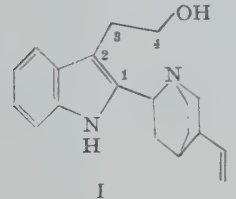
[2] For the best review see P. L. JULIAN, E. W. MEIER, and H. C. PRINTY, in *Heterocyclic Compounds*, Vol. III (John Wiley & Sons, Inc., New York, 1952), Chapter 1, pp. 161 and 225.

[3] P. L. JULIAN, H. C. PRINTY, R. KETCHAM, and R. DOONE, J. Amer. Chem. Soc. *75*, 5305 (1953).

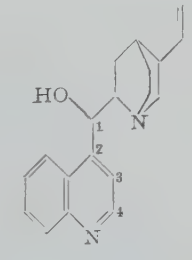
[1] It is interesting to note that the cyclization of compounds containing two hetero atoms results in the formation of 5-membered lactams; e.g.:

[P. W. NEBER, Ber. dtsch. chem. Ges. *55*, 826 (1922). – P. W. NEBER and H. KEPPLER, Ber. dtsch. chem. Ges. *57*, 778 (1924). – J. MARTINET and O. DORNIER, C. r. Acad. Sci. *172*, 1415 (1921). – F. J. DI CARLO, J. Amer. Chem. Soc. *66*, 1420 (1944)].

[2] P. L. JULIAN, E. W. MEIER, and H. C. PRINTY, Note 1, p. 418.



I



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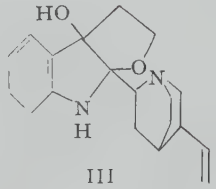

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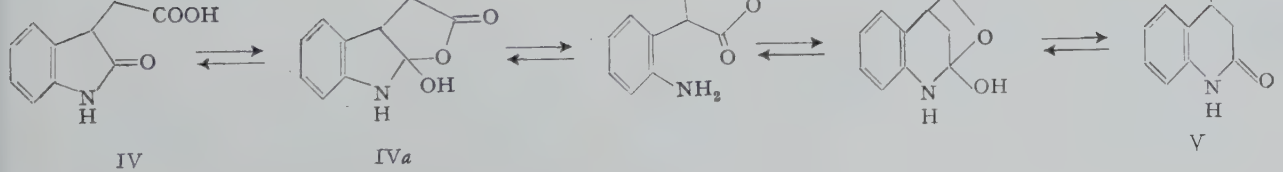
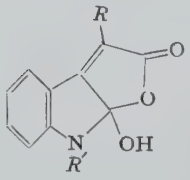
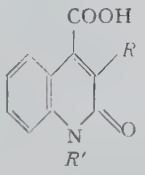


IV IVa V

The presence of steric factors which may restrict the rotation of the side-chain carboxyl group and hold it rigidly in close proximity to the lactam linkage would diminish the stability of the oxindole nucleus. Such is the case with substituted carboxymethyleneoxindoles[1]. The base-or acid-catalyzed condensation products of isatin with malonic acid derivatives (after hydrolysis), malonic acid itself, or phenylacetic acid, and the Reformatsky reaction products of variously N-substituted isatins have been proved to possess the 1- and/or 3-



VI



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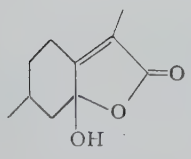
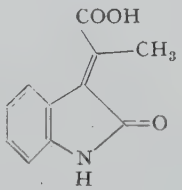


VIII



IX

substituted 2-hydroxycinchoninic acid structure (VII). The short-lived intermediate carboxymethylene compounds would be expected to exist exclusively in their pseudo-acid form (VI). The latter has an excellent analogue in the recently assigned structure of the autoxidation product of menthofuran (VIII)[2]. Internal interaction as illustrated in VI can be expected only in stereoisomers whose carboxyl and amide groups are cis toward each other, i.e. in compounds whose *R* group is equal to or larger than the carboxyl group. Thus the stability of the carboxymethylene compound most recently obtained by the base-catalyzed reaction of oxindole and pyruvic acid or its acetimino derivative[3] is not surprising. The position of the carboxyl group in this product must be in a trans relationship to the lactam function (*vide* IX).

[1] P. L. Julian, E. W. Meier, and H. C. Printy in *Heterocyclic Compounds*, Vol. III (John Wiley & Sons, Inc., New York, 1952), Chapter 1.

[2] R. B. Woodward and R. H. Eastman, J. Amer. Chem. Soc. *72*, 399 (1950).

[3] P. L. Julian, H. C. Printy, R. Ketcham, and R. Doone, J. Amer. Chem. Soc. *75*, 5305 (1953).

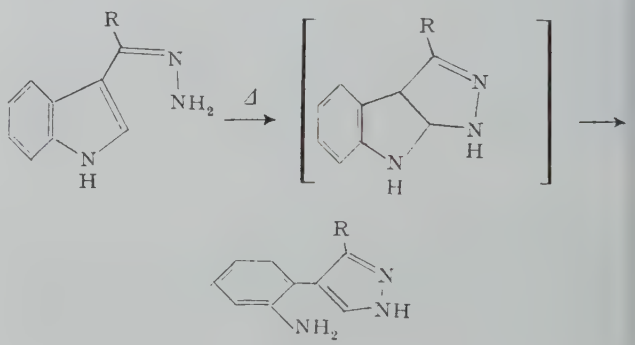
Intramolecular reactions between a hetero atom at the end of a C_3-situated two-carbon sidechain and the lactam or imine functions of oxindoles or indolenines, respectively, are quite common. Hence the formation of tetrahydrofuro- and tetrahydropyrrolo-indoles through such reactions as peracid oxidation of indoles, e.g. Witkops's conversion of cinchonamine (I) to quinamine (III)[1], alkylation of tryptamine- or tryptophol-Grignard complexes[2], and chemical reduction of substituted oxytryptamines, e.g. Julian's synthesis of physostigmine[3], indicate the generality of this interaction. An interesting similar case involves the conversion of 3-phenylacetyloxindole oxime (X) to 3-phenylethylindole (XII) on catalytic hydrogenation[4]. Acid-induced cyclization to an isoxazole followed by hydrogenolysis could be envisaged to yield 3-phenylacetylindole oxime (XI), which in turn would undergo hydrogenation and hydrogenolysis producing the indole (XI)[5]. (Form. X, XI, XII).

E. Wenkert and Th. L. Reid

Department of Chemistry, Iowa State College, Ames, Iowa, December 21, 1953.

Zusammenfassung

Es werden verschiedene Reaktionen in der Oxindolreihe diskutiert. Man kann sie sich als ähnliche Mechanismen vorstellen, da alle über intramolekular gebildete Zwischenprodukte bzw. Endprodukte laufen.



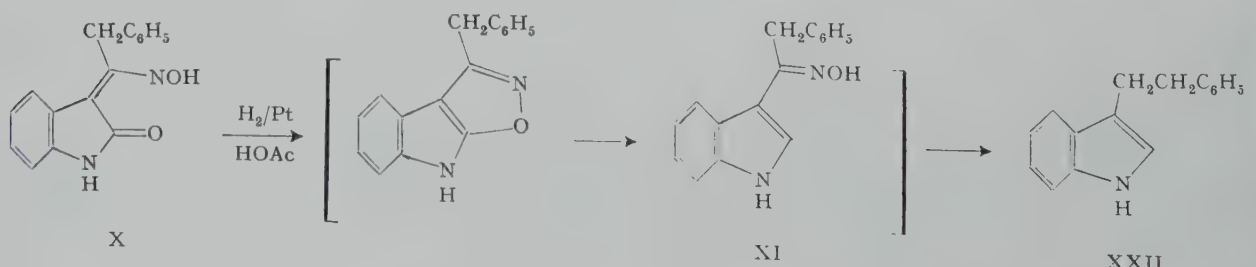
[1] B. Witkop, J. Amer. Chem. Soc. *72*, 2311 (1950).

[2] T. Hoshino *et al.*, Ann. Chem. *500*, 42 (1932); *520*, 19 (1935).

[3] P. L. Julian and J. Pikl, J. Amer. Chem. Soc. *57*, 539 (1935).

[4] R. B. Woodward and E. Wenkert, unpublished results. The authors express their thanks to Professor Woodward for permission to include the above data in this article.

[5] As a sidelight to the above discussion, the interesting thermal rearrangement of 3-acylindole hydrazones should be noted [C. Alberti, Gazz. Chim. ital. *77*, 398 (1947)]. This reaction may be postulated to proceed via an internally interacted intermediate which breaks up into the more thermodynamically stable aminophenylpyrazole:



X

XI

XXII

The Reaction of *trans*-Decahydroquinoline with Molecular Oxygen [1]

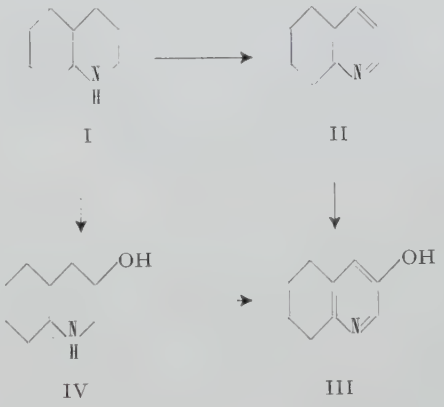
When a steady stream of oxygen was bubbled through *trans*-decahydroquinoline (m.p. 48°) at a temperature of 80–100° for a period of ten and more hours, no traces of peroxidic material were detectable at any time. In contrast to the oxygenation of *cis*-[2] and *trans*-decalin[3] any hydroperoxide initially formed in this case would probably be converted to the corresponding carbinol because of the secondary amino group present in the molecule[4].

The reaction mixture was separated into phenolic, basic and neutral fractions. The basic part consisted of starting material and 4–8 % of a much weaker base, viz., 5,6,7,8-tetrahydroquinoline, isolated as the picrate, m.p. 158°[5]. Found: C, 49·67 %; H, 3·93 %; N, 15·64 %. Calculated for $C_9H_{11}N \cdot C_6H_3N_3O_7$: C, 49·73 %; H, 3·87 %; N, 15·46 %. Oxygen thus has effected a dehydrogenation of the heterocyclic moiety of the bicyclic system in accordance with the course of the dehydrogenation of *cis*- and *trans*-decahydroquinoline[6] and -isoquinoline[7]. The conversion of N-methylyohimbane to the cation of N-methyltetradehydroyohimbane by the action of catalytically excited oxygen at room temperature[8] is a related case.

The phenolic compound formed transparent prisms from ether, m.p. 198–200°. Found: C, 68·08 %; H, 7·45 %; N, 8·58 % (compound dried at room temperature). Calculated for $C_9H_{11}NO \cdot 1/2H_2O$: C, 68·33 %; H, 7·65 %; N, 8·85 %. The picrate crystallized from methanol in tufts of yellow needles, m.p. 208–211°C. Found: C, 47·88 %; H, 3·81 %; N, 14·47 %. Calculated for $C_9H_{11}NO \cdot C_6H_3N_3O_7$: C, 47·62 %; H, 3·73 %; N, 14·81 %.

The composition, the phenolic character and the similarity of the ultraviolet absorption data with those of 3-hydroxypyridine[9] (Table) suggest the structure of the hitherto unknown 3-hydroxy-5,6,7,8-tetrahydroquinoline (III). The *bathochromic* shift observed in the ultraviolet spectrum on the formation of the anion (or cation) is typical of phenolic compounds and rules out the analogous 2- and 4-tetrahydroquinolones which would show a *hypsochromic* shift[1].

The neutral fraction contained at least two different compounds so far not obtained crystalline but characterized by infrared data. The fraction more easily eluted from Al_2O_3 by benzene containing 1 % chloroform showed (in $CHCl_3$) carbonyl bands at 5·84 and 6·15 μ. The subsequent fraction, distillable at 170°/0·3 mm showed (in $CHCl_3$) a weak band at 2·94 (>NH), a sharp and strong band at 6·05 and 6·90 (>CO-NH), indicating a compound possibly related to the known 5,6,7,8-tetrahydro-[2] or *trans*-octahydrocarbostyril[3], excluding carbostyril (bands at 3·0; 6·06[s]; 6·24[m]; 6·44[m]; no band at 6·90) and 1,2,3,4-tetrahydrocarbostyril (2·98; 5·99[s]; 6·30[s]; 6·80[s]). However, the absence of secondary amide bands in the 6·5 μ region more or less eliminates any (hydrogenated) carbostyril structures; the two neutral fractions are rather the products of a complicated sequence of oxidation and rearrangement reactions to be reported elsewhere (ref. [4]).



The phenolic 3-hydroxy-5,6,7,8-tetrahydroquinoline (III) is probably formed by further oxidation of the parent II rather than by dehydrogenation of the hypo-

[1] Oxidation Mechanisms. XIV. Preceding paper: B. Witkop and S. Goodwin, J. Amer. Chem. Soc. *76*, 1954, in press.
[2] R. Criegee, Ber. dtsch. chem. Ges. *77*, 22, 722 (1944).
[3] H. Kleinfeller and C. Odefey, Angew. Chem. *62*, 342 (1950).
[4] *Cf.* C. W. Capp and E. G. E. Hawkins, J. Chem. Soc. *1953*, 4106.
[5] M. Ehrenstein and W. Bunge, Ber. dtsch. chem. Ges. *67*, 1715 (1934).
[6] J. v. Braun and G. Lemke, Liebigs Ann. Chem. *478*, 191 (1930). – M. Ehrenstein and W. Bunge, Ber. dtsch. chem. Ges. *67*, 1715 (1934).
[7] B. Witkop, J. Amer. Chem. Soc. *70*, 2617 (1948).
[8] B. Witkop, J. Amer. Chem. Soc. *75*, 3361 (1953).
[9] H. Specker and H. Gawrosch, Ber. dtsch. chem. Ges. *75*, 1347 (1942). – *Cf.* T. R. Govindachari and N. S. Narasimhan, J. Chem. Soc. *1953*, 2635.

[1] H. Specker and H. Gawrosch, Ber. dtsch. chem. Ges. *75*, 1347 (1942). – *Cf.* T. R. Govindachari and N. S. Narasimhan, J. Chem. Soc. *1953* 2635.
[2] H. K. Sen-Gupta J. Chem. Soc. *107*, 1357 (1915); *cf.* A. Dornow and E. Neuse, Ber. dtsch. chem. Ges. *84*, 296 (1951).
[3] W. Hückel and F. Stepf, Liebigs Ann. Chem. *453* 168 (1927).
[4] L. A. Cohen and B. Witkop, J. Amer. Chem. Soc. (in prep.)

Solvent	Phenolic Oxidation Product of *trans*-Decahydroquinoline		Solvent	3-Hydroxypyridine[1]	
	λmax (log ε)	$\Delta\lambda$		λmax (log ε)	$\Delta\lambda$
EtOH	292 (3·71)	–	MeOH	277 (3·61)	–
0·1*N*-HCl-EtOH	303 (3·84)	+11	0·1*N*-HCl-MeOH	284 (3·81)	+ 7
0·1*N*-KOH-EtOH	315 (3·72)	+22	0·1*N*-KOH-MeOH	301 (3·62)	+24

Absorption maxima of the phenolic oxidation product from I and of 3-hydroxypyridine; $\Delta\lambda$ signifies the bathochromic shift when going from the free base (or zwitterion) to the cation or anion.

[1] H. Specker and H. Gawrosch, Ber. dtsch. chem. Ges. *75*, 1347 (1942). – *Cf.* T. R. Govindachari and N. S. Narasimhan, J. Chem. Soc. *1953*, 2635.

thetical 3-hydroxydecahydroquinoline (IV). The former course of oxidation is analogous to the introduction of β-hydroxy groups into pyridine[1] and quinoline[2] on biological oxidation. The latter route would be comparable to the β-oxygenation of *trans*-decaline (→ β-decalone)[3]. The possible introduction of a hydroxy group in the 2-position is reminiscent of the oxidation of niacine[4] and quinine[5].

By contrast, $\Delta^{1(9)}$-octahydroquinoline, being a tertiary unsaturated amine with only one activated tertiary center for the attack of oxygen, easily forms, as do similar bicyclic systems of this type a beautifully crystalline hydroperoxide[6].

B. Witkop

National Institutes of Health, Washington 14, D.C., April 21, 1954.

Zusammenfassung

Dem phenolischen Oxydationsprodukt, das beim Durchleiten von Sauerstoff durch geschmolzenes *trans*-Dekahydrochinolin entsteht, wird auf Grund der Analyse und spektrophotometrischen Daten die Konstitution III eines 3-Oxy-5, 6, 7, 8-tetrahydrochinolins beigelegt. Seine Entstehung erfolgt wahrscheinlich über das gleichfalls gebildete 5, 6, 7, 8-Tetrahydrochinolin.

[1] *Cf.* J. N. Smith and R. T. Williams, Biochem. J. *56*, 325 (1954).
[2] L. Novack and B. B. Brodie, J. Biol. Chem. *187*, 787 (1950).
[3] H. Kleinfeller and C. Odefey, Angew. Chem. *62*, 342 (1950).
[4] *Cf.* M. E. Pullman and S. P. Colowick, J. Biol. Chem. *206*, 121 (1954).
[5] B. B. Brodie, J. E. Baer, and L. C. Craig, J. Biol. Chem. *188*, 567 (1951).
[6] L. A. Cohen and B. Witkop, J. Amer. Chem. Soc. (in preparation).

Spectrophotometric Differences between Aminoheterocyclic Bases and Their Salts

When an open or cyclic base of the Schiff type containing the element >C=N– passes into the cation $>C=\overset{\oplus}{N}H-$ three major spectroscopic changes are observed in the ultraviolet and infrared absorption spectra:

(1) A bathochromic shift in the ultraviolet ranging between 1 and 50 mμ and more depending on the type of compound and the presence of auxochromic groups[1]. A hypsochromic shift on salt formation usually indicates the participation of the cation $>C=\overset{\oplus}{N}H-$ in partial or complete *intra*- or *inter*molecular addition reactions[2]. Pyridine and its derivatives are not normally looked upon as cyclic Schiff bases, although a number of chemical reactions (1, 2-addition and reduction, such as addition of alkyl lithium, Hammick reaction, acyloin-like condensations with aldehydes, etc.) clearly indicate the independence of the "ammono-aldehyde" (Morton) system. In other respects pyridine exhibits aromatic character. This dualistic behavior is reflected in the effect of salt formation on the ultraviolet absorption of pyridines which may vary from hypsochromic to bathochromic (Table I).

[1] *Cf.* B. Witkop, J. B. Patrick, and H. M. Kissman, Ber. dtsch. chem. Ges. *85*, 949 (1952).
[2] *Cf.* E. D. Bergmann, Chem. Rev. *53*, 309 (1953).

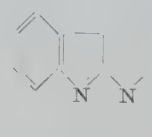
Table I

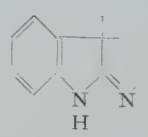
Influence of Salt Formation on the Ultraviolet Absorption of Some Pyridines (solvent ethanol if not stated otherwise).

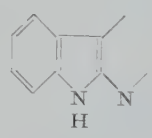
	λ_{max} free base	λ_{max} salt	$\Delta\lambda$
Pyridoxamine	308 (3·86)[a]	293 (3·95)	−15
Pyridoxal	300 (3·76)	288 (3·93)	−12
Pyridine[b]	257 (3·43)[c]	256 (3·73)	− 1
Nicotine[b]	262 (3·46)	260 (3·68)	− 2
2,6-Lutidine[b]	267 (3·48)[d]	272 (3·68)	+ 5
3-Vinylpyridine[b] . . .	278 (3·44)	287 (3·53)	+ 9
Nicotyrine[b]	288 (3·99)	310 (4·04)	+22

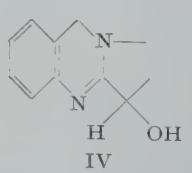
[a] Measured in 0·1 *N* NaOH: A. Meister, E. A. Peterson, and H. A. Sober, J. Amer. Chem. Soc. *76*, 169 (1954). [b] M. L. Swain, A. Eisner, C. F. Woodward, and B. A. Brice, J. Amer. Chem. Soc. *71*, 1341 (1949), measured in 95% alcohol. [c] *Cf.* H. V. Daeniker, Helv. chim. Acta *35*, 1955 (1952). [d] Measured in isooctane: R. A. Friedel and M. Orchin, *Ultraviolet Spectra of Aromatic Compounds* (John Willey and Sons, Inc., New York, 1951), p. 106. – F. G. Herington, Discussions of the Faraday Society *9*, 26 (1950).

α- and γ-aminopyridines are no longer formulated as α- or γ-pyridone imines but as cyclic (vinylogous) amidines; the true N-methyl pyridone imines which are only present in anhydrous inert solvents, absorb at much longer wave length than their tautomeric amidine cations; in this respect the salt formation has a hypsochromic effect. A slight hypsochromic effect is also observed when forming the salt of α-aminoindolenine, the properties of which (Table III) are best explained by formula I rather than II[1] or III[2], or of a cyclic amidine where the amino group forms part of a second ring as in vasicine (peganine, IV, Table III).


I


II


III

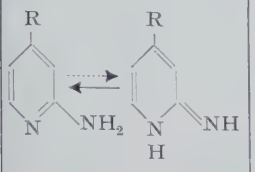
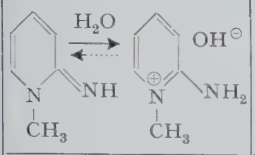
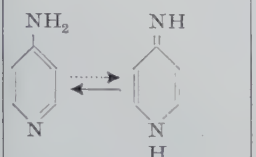
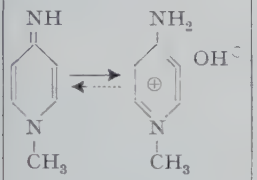
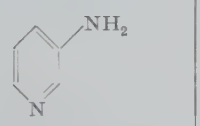
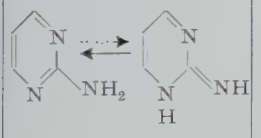

IV

(2) The infrared absorption of the >C=N– group (6·10–6·15 in aromatic Schiff bases, 6·24–6·28 in pyridines[3]) on salt formation invariably moves to shorter wave length (5·98–6·08 in Schiff bases, 6·07–6·13 in pyridines). This hypsochromic shift is even stronger in open and cyclic amidines (Table II and III) and clearly

[1] H. Rinderknecht, H. Koechlin, and C. Niemann, J. Org. Chem. *18*, 971 (1953).
[2] R. Pschorr and G. Hoppe, Ber. dtsch. chem. Ges. *43*, 2543 (1910).
[3] The assignment of the band 6·28 μ (1590 cm^{-1}) to the group >C=N– in pyridines (H. M. Randall, R. G. Fowler, N. Fuson, and J. R. Dangl, *Infrared Determination of Organic Structures* [D. Van Nostrand Co., New York, 1949], p. 32) or pyrimidines (*Cf.* I. A. Brownlie, J. Chem. Soc. *1950* 3062), or other heterocycles is a simplification convenient for the purpose of comparison with the hydrochlorides. Normally no absolute assignments to >C=C< or >C=N– in conjugated systems can be made with certainty (*Cf.* E. R. Blout, M. Fields, and R. Karplus, J. Amer. Chem. Soc. *70*, 194 [1948]).

Table II

The Effect of Salt Formation on the UV and IR Absorption Spectra of Aminopyridines and -Pyrimidine.

Tautomers	Compound (measured in N = Nujol, or C = Chloroform)	IR-Absorption: Immonium Bands	IR-Absorption: >C=N–	IR-Absorption: aromatic	Basicity pK_A	UV-Absorption λ_{max}	$\Delta\lambda$ λ_{max} cation $\overset{\oplus}{(>C=NH-)}$ λ_{max} base (>C=N–): IR (μ)	$\Delta\lambda$ λ_{max} cation $\overset{\oplus}{(>C=NH-)}$ λ_{max} base (>C=N–): UV (μ)
	α-aminopyridine (R = H) (C)	—	6·17[s] (6·18)	6·38[m a] (6·41)	6·86[b]	295[c]	—0·20	+5
	α-aminopyridine hydrochloride (N)	—	5·98[s]	6·11[s a]		300[c]		
	1-methyl-1,2-dihydro-2-iminopyridine (C)	—	6·05[s]	6·37[s e]	12·20[b]	353 (3·48)[d] in ether	—	~—50
	1-methyl-2-amino-pyridinium hydroxide (in H_2O)	—				303 (3·65)[d] in aqueous diox.		
	γ-aminopyridine (C)	—	6·14[s]	6·24[s e]	9·17[b]	245[c]	—	+19
	γ-aminopyridine hydrochloride	—				264[c]		
	1,4-dihydro-4-amino-1-methylpyridine (C)	—	6·04[s]	6·48[s e]	12·5[b]	315 (3·46)[d] in aqueous diox.	—	~—43
	1-methyl-4-amino-pyridinium hydroxide (in H_2O)	—				272 (4·45)[d]		
	3-aminopyridine (C)	—	6·16[s] (6·16)	6·29[s f] (6·31) 6·15[s]	6·6[c]	298 (3·59)ether[d] 304 (ethanol)[e]	—0·10	~+12
	3-aminopyridine monohydrochloride (N)	—	6·06[s]	6·22[m]			—0·06	
	3-aminopyridine dihydrochloride (N)	4·80; 4·92; 5·14	6·10[m]	6·45[s]		316[c]		
	2-aminopyrimidine (C)	—	— 6·02[s]	6·23[s f] 6·19[s]	3·54[h]	292 (3·50)	—	~+11
	2-aminopyrimidine hydrochloride (N)	—	6·02[s]	6·19[s]		303 (3·60)		

[a] The infrared data are those of the homologous 4-methyl-α-aminopyridine. The figures given below in parentheses are those of 2-aminopyridine observed by S. J. Angyal and R. L. Werner, J. Chem. Soc. *1952*, 2911. [b] *Cf.* S. J. Angyal and C. L. Angyal, J. Chem. Soc. *1952*, 1461. [c] E. A. Steck and G. W. Ewing, J. Amer. Chem. Soc. *70*, 3397 (1948). [d] L. C. Anderson and N. V. Seeger, J. Amer. Chem. Soc. *71*, 340 (1949); 1-methyl-2-pyridonemethide absorbs at considerably longer wave length: 422 (3·18); 277 (3·25). [e] S. J. Angyal and R. L. Werner, J. Chem. Soc. *1952*, 2911. [f] The figures in parantheses are those of S. J. Angyal and R. L. Werner, J. Chem. Soc. *1952*, 2911. [g] D. J. Brown and L. N. Short, J. Chem. Soc. *1953*, 331; $\Delta\lambda$ decreases on methylation: 2-methylaminopyrimidine $\Delta\lambda$ + 8 – 9; 2 dimethylaminopyrimidine + 6 – 7 mμ. The three analogous compounds in the 4-aminopyrimidine show the following negative $\Delta\lambda$: 4-aminopyrimidine — 22–23; 4-methylaminopyrimidine — 22–23; 4-dimethylaminopyrimidine — 26. [h] A. Albert, R. Goldacre, and J. Phillips, J. Chem. Soc. *1948*, 2240.

distinguishes the cation of α- and γ-aminopyridine from the (double) cation of β-aminopyridine. The lowest absorption so far observed occurred in certain guanidinium cations, e.g., 5·65[s] and 5·64[s] in creatinine hydrochloride (measured in nujol).

(3) The infrared absorption of the cation $>C=\overset{\oplus}{N}H-$ always shows the broad ammonium band at 4·0–4·3 μ and one or several bands in the triple bond region 4·5–5·5 μ. These bands, which may be termed *immonium bands*, are characteristic of the salts of open and cyclic imines including heterocycles such as pyridine, quinoline, isoquinoline, etc. The neighborhood of a heteroatom of small basicity, e.g., oxygen, weakens the extinction of the immonium band, e.g., in benz-1,3-oxazines, or suppresses it completely as in acetiminoether hydrochloride. The presence of a second basic heteroatom, such as in the hydrochlorides of open or cyclic amidines, leads to the complete disappearance of these immonium bands. This observation is of diagnostic value, as can be seen from the comparison of the three aminopyridines: only the *di*hydrochloride of β-aminopyridine shows

Table III

The Effect of Salt Formation on the UV and IR Absorption Spectra of α-Aminoindolenine (I) and of Alkaloids of Partly Unknown Structure

Compound	Infrared Absorption				Basicity (pK_A and pK'_A)	Ultraviolet data (in neutral EtOH or N/10 ethanolic HCl)	$\Delta\lambda$ λ_{max} cation $\overset{\oplus}{(}$>C=NH—Ph) λ_{max} base (>C=NPh)	
	OH or NH	–C=N–	Aromatic	$CHCl_3$ (C) or Nujol (N)			IR (μ)	UV (mμ)
α-Aminoindolenine (I)	2·85 2·95 3·04	6·13[s]	6·24[s] 6·22[w]	C N	~8·0 (R = H)[a] [~10·0 (R = Me)[a]]	[289 (3·50)] 267 (4·05)	—0·08	+1 (—7)
α-Aminoindolenine hydrochloride	(2·98)	5·93[s] 6·13[s]	6·24[s]	C	—	290 (3·40) 260 (3·96) 252 (3·95)		
Quebrachamine (*cf.* III)	2·88	—	—	C	6·76 (80% methyl cellosolve)	295 (3·85) 286 (3·88)	—	—5
Quebrachamine hydrochloride	3·15	—	6·18[s]	N	9·25 (75% EtOH)	290 (3·83) (methiodide)		
β-Hydroxyquebramidine (*cf.* I)[f]	2·79	—[e]	6·20[w]	C	11·0[d] (80% methyl cellosolve)	293 (3·52) 235 (3·96)	—	± 0
β-Hydroxyquebramidinehydrochloride	3·15	—[e]	6·18[s]	N	10·57[d] (7·43) (in H_2O)	293 (3·39) (methiodide or hydriodide)		
nor-C-Curarine-I (*cf.* I)	—	6·05[s]	6·25[s]	C	6·26 (EtOH)[b] 8·5 (H_2O)[b]	302 (3·75) (in alcoholic KOH)[c]	—0·01	—12
nor-C-Curarine-I hydrochloride	2·93	6·04[s]	6·23[s]	C	—	290 (3·68) 257 (3·87)		
Vasicine (peganine) IV	—	6·10[s]	6·23[s]	C	—	304 (3·66)	—0·20	—19
Vasicine hydrochloride	3·2	5·90[s]	6·15[m]	N	—	285 (3·51)		

[a] Private communication from Dr. S. J. Angyal, *cf.* S. J. Angyal, Australian J. Scient. Res. [A] *5*, 377 (1952). I am greatly indebted to Dr. Angyal for this communication and for a stimulating discussion. [b] P. Karrer and H. Schmid, Helv. chim. Acta *29*, 1558 (1946). [c] H. Schmid, A. Ebnöther, and P. Karrer, Helv. chim. Acta *33*, 1487 (1950). [d] These pK values are of limited value, since chemical changes (double bond tautomerism or hydrolysis) seem to occur during the titration. [e] Another indolenine of this type, 3-hydroperoxy-3-methyl-2-phenyl-(or 2-*p*-methoxyphenyl)-indolenine likewise lacks absorption in this region: B. Witkop, J. B. Patrick, and H. M. Kissman, Ber. dtsch. chem. Ges. *85*, 949 (1952). [f] *Cf.* B. Witkop, Bull. Soc. Chim. France *1954*, 423.

immonium bands (Table II) but not the hydrochlorides of α-, β-, γ-aminopyridine or of α-aminoindolenine. Alkaloidal hydrochlorides can be assayed by this method (Table IV).

The hydrochlorides of the alkaloids listed in Table III do not show immonium bands. If β-hydroxyquebramidine and *nor*-C-cuarine-I hydrochlorides be accepted as indolenines, as a number of chemical and spectrophotometric indications seem to suggest[1], the absence of

[1] *Cf.* B. Witkop and J. B. Patrick, J. Amer. Chem. Soc. *75*, 4474 (1953); Angew. Chem. *65*, 467 (1953).

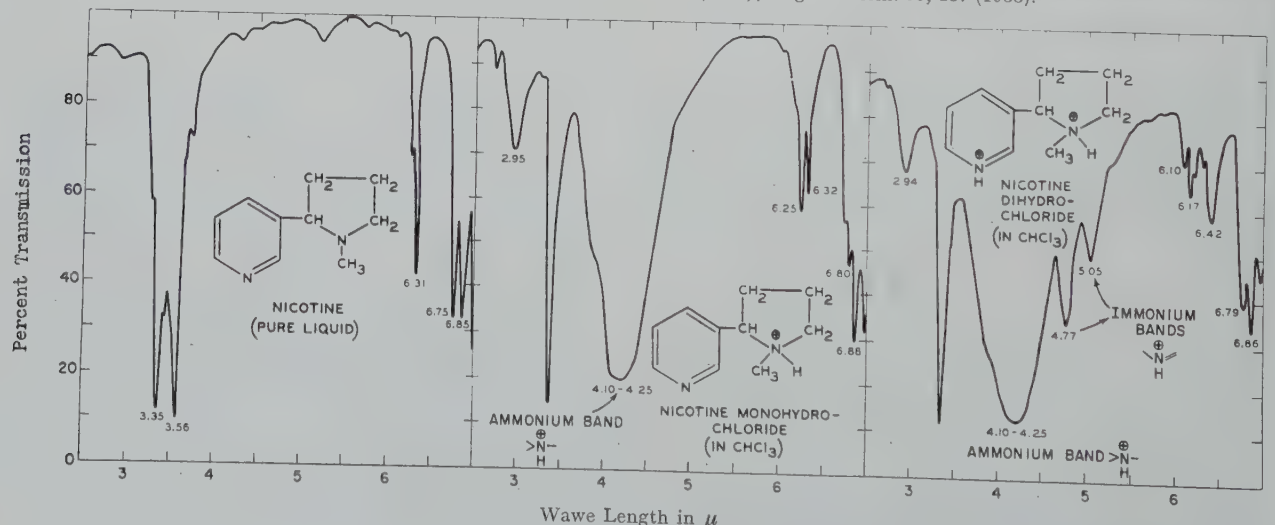


Fig. 1.—The effect of stepwise salt formation on the infrared spectrum of an alkaloid with a saturated and an unsaturated tertiary nitrogen function.

Table IV

Assay of Variouy Hydrochlorides for the Presence of "Immonium Bands"

Compound (measured in C = Chloroform, or N = Nujol)	Immonium Bands
Nicotine monohydrochloride (Fig. 1 (C)	–
Nicotine dihydrochloride (Fig. 1) (C) . .	4·77^{m}; 5·05^{m}
Peganine (vasicine) hydrochloride (N) .	–
Pegadiene hydrochloride (C)	–
4-Quinazolone hydrochloride (N)[a] . . .	4·92^{vw}; 5·30^{vw}
Febrifugine dihydrochloride (N)[a] . . .	4·86^{w}; 5·14^{m}; 5·43^{m}

[a] It was expected that the weakly basic character of the amide nitrogen in position 3 would restore the independence of the cation

$$\underset{1}{\text{NH}}=\underset{2}{\text{CH}}-\underset{3}{\text{NH}}-\underset{4}{\text{CO}}$$

by suppressing the contribution

$$\underset{1}{\text{NH}}-\underset{2}{\text{CH}}=\underset{3}{\text{NH}}-\underset{4}{\text{CO}}.$$

Such an effect is operative and leads to the appearance of immonium bands, very weak in quinazolone hydrochloride, more distinct in the dihydrochloride of the quinazolone alkaloid febrifugine (an alkaloid from Hydrangea, *cf.* B. R. Baker, *et al.*, J. Org. Chem. *17*, 132 [1951]; I am greatly indebted to Dr. Baker for samples), where there must be bonded or non-bonded interaction between the three oxygen functions: desoxyfebrifugine dihydrochloride, where no such interaction is possible, lacks immonium bands.

immonium bands is unexpected and might be due to the direct or, less likely, vinylogous attachment of N^{b} to the $>C=N^{a}-$ group, in other words, an α-aminoindolenine type of structure (I). The substantiation of this concept is awaiting further chemical experiments. A full report on further uses of the immonium bands for diagnostic purposes (positional effect of alkyl substituents in pyridines and quinolines, shift to longer wave length in aromatic N-oxide hydrochlorides, ketimine-enamine tautomerism, etc.) will appear in the near future[1].

B. Witkop

National Institutes of Health, Washington 14, D.C., February 8, 1954.

Zusammenfassung

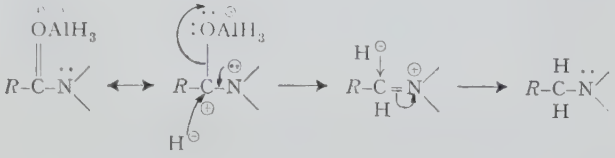
Bei der Salzbildung offener oder zyklischer Schiffscher Basen, eingeschlossen aromatische Heterozyklen, wie Pyridin, Chinolin, Benzoxazin, Indolenin usw., können die im UV.- und UR.-Spektrum auftretenden batho- oder hypsochromen Verschiebungen sowie das Auftreten oder Ausbleiben der für das Kation $>C=\overset{\oplus}{N}H-$ charakteristischen «Immonium-Banden» bei 4,5–5,5 μ zur Erkennung der Natur und der Position von Aminosubstituenten benutzt werden.

[1] The method has been utilized to advantage in the meantime: the tobacco alkaloid myosmine was found to be a Δ_1-pyrroline derivative as the free base as well as in the form of the mono- and dihydrochloride, B. Witkop, J. Amer. Chem. Soc. *76*, in press. Likewise, new structures had to be postulated for tetrahydropyridine and γ-coniceine (added in proof).

The Reduction of Amides with Lithium Aluminium Hydride[1]

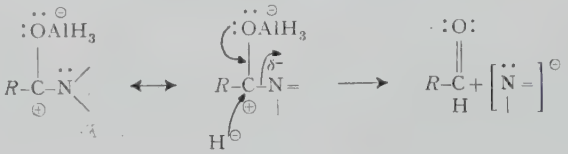
The reduction of carboxylic acid amides with lithium aluminium hydride generally yields the corresponding amine with the same number of carbon atoms[2]. However, in some cases, amides are cleaved to alcohols or aldehydes and amines[3].

The following mechanism has been proposed[4] for the reduction of amides and vinylogs of amides to yield the corresponding oxygen-free compounds:



The reactive species here is the aluminohydride ion $AlH_4^{\ominus}$ which, according to Paddock[5], exists in ether solution in equilibrium with aluminium hydride and the hydride ion. The attack of the hydride ion and the tendency of the nitrogen atom to donate its electron pair to the electron deficient carbon results in a cleavage of the carbon-oxygen bond to yield the amine, with a total consumption of one-half mole of lithium aluminium hydride.

When the electron-donating tendency of the nitrogen atom is decreased by stabilization, the adjacent carbon assumes a more positive character. The attack by the hydride ion alone or in conjunction with a shift of electrons from the oxygen atom results in a cleavage of the carbon-nitrogen bond to yield the carbonyl derivative and the starting amine.



The isolation of aldehydes generally requires the addition of one-quarter mole of lithium-aluminium hydride to a solution of amide. These conditions are fulfilled by the indicated mechanism. The aldehyde therefore does not arise as a result of the hydrolysis of an intermediate complex, as has been postulated[6], but is present in the reaction mixture prior to hydrolysis. The use of excess complex hydride causes a reduction of the aldehyde to the corresponding alcohol.

The mechanism has been experimentally verified. The benzamide of benzotriazole was treated with one-quarter mole of lithium aluminium hydride in ether, with inverse

[1] Contribution No. 13 from the Yerkes Research Laboratory.

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[4] N. G. Gaylord, Exper. *10*, 166 (1954).

[5] N. L. Paddock, Nature *167*, 1070 (1951).

[6] V. M. Mićović and M. L. Mihailović, J. Org. Chem. *18*, 1190 (1953). – M. Mousseron, R. Jacquier, M. Mousseron-Canet, and R. Zagdoun, Bull. Soc. chim. France [5] *19*, 1042 (1952).

addition. The infrared spectrum of a sample of the reaction mixture, withdrawn before hydrolysis, indicated a strong absorption at 5·85 μ, and was identical, over the range 5·75–6·00 μ, to the spectrum obtained from a synthetic mixture containing benzotriazole, the benzamide of benzotriazole, benzaldehyde and ether, and was different from that obtained from the synthetic mixture in the absence of benzaldehyde. The reduction products, after hydrolysis and the usual work-up, were benzaldehyde and benzotriazole. It has previously been reported that the reduction of the benzamide of benzotriazole with excess lithium aluminium hydride yields benzyl alcohol and benzotriazole[1].

A nitrogen-aluminium bond has been postulated as an intermediate in the cleavage of compounds containing the $-\overset{\cdot\cdot}{\underset{\cdot\cdot}{N}}-\overset{|}{\underset{|}{C}}-\overset{\cdot\cdot}{\underset{\cdot\cdot}{O}}-$ grouping[2]. The failure to form an oxygen-aluminium bond in such compounds may be due to the non-polarizability of this carbon-oxygen bond.

N. G. Gaylord

Yerkes Research Laboratory, Film Department, E. I. du Pont de Nemours and Company, Inc., Buffalo 7, N.Y., June 30, 1954.

Zusammenfassung

Ein einfacher Mechanismus wurde vorgeschlagen und ein experimenteller Beweis gegeben, um die Bildung von Aminen oder Abspaltungsprodukten in der Reduktion von Amiden mit Lithiumaluminiumhydrid zu erklären.

[1] N. G. Gaylord, J. Amer. Chem. Soc. *76*, 285 (1954).
[2] N. G. Gaylord, Exper. (in press).

Infra-red Study of the Carbonate in Bone, Teeth and Francolite[1]

Introduction

One of the major problems in the crystallography of the inorganic portion of teeth and bone, as well as the mineral francolite, is the elucidation of their carbonate content. Many investigators have claimed that the carbonate is found as an amorphous calcium and magnesium carbonate physically mixed with the apatite phase of these substances[1]. There are others who believe that a carbon radical substitutes in some way, in a structural position of the apatite, forming a carbonate-bearing member of the apatite series[2].

The amount of carbon dioxide released by treating these materials with acid is by no means a trace amount. In the four samples studied, we have the following analyses based on samples purified by specific gravity separation and dried at 110°C. (It is well to note that the bone, dentine and enamel were freed of organic material by boiling in a solution of KOH in glycerine according to the method of Gabriel[3].)

Sample	% CO_2
cow enamel	2·88
cow dentine	3·91
cow bone (femur)	5·15
francolite	2·70

X-ray work

It is evident that if the CO_2 were present as well crystallized $CaCO_3$ and $MgCO_3$, it would be possible to see the lines of these materials by X-ray diffraction. In fact it was demonstrated that it is possible to detact as little as 3% $CaCO_3$ admixed with apatite by X-ray diffraction methods[4]. However, the latter proof depends upon well crystallized calcite and in the case of the materials studied, it is postulated that the carbonates are too finely crystallized to be detected by X-ray

[1] This work was partially sponsored by the *Air Research and Development Command*, United States Air Force, through its European Office under contract n° AF 61 (514) 647 C.

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[2] D. McConnell and J. W. Gruner, Amer. Min. *25*, 157 (1940). – D. McConnell, Bull. Soc. franç. Minéral. Crist. *76*, 428 (1952).
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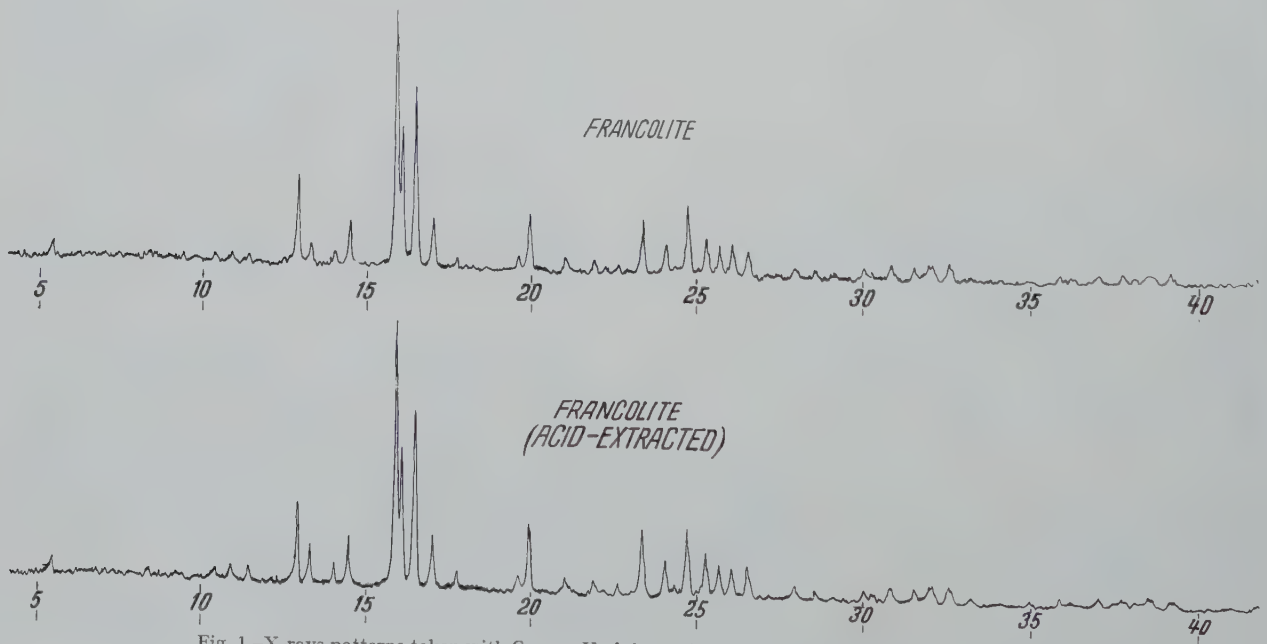

Fig. 1.–X-rays patterns taken with Copper K-alpha radiation. Angles given in degrees theta (Θ).

methods. The presence of particles in the inorganic portion of bone and teeth which range in size below 100 Å has been demonstrated by low angle X-ray scattering techniques[1]. However this technique does not tell us exactly what crystalline phase is in that size range. Thus

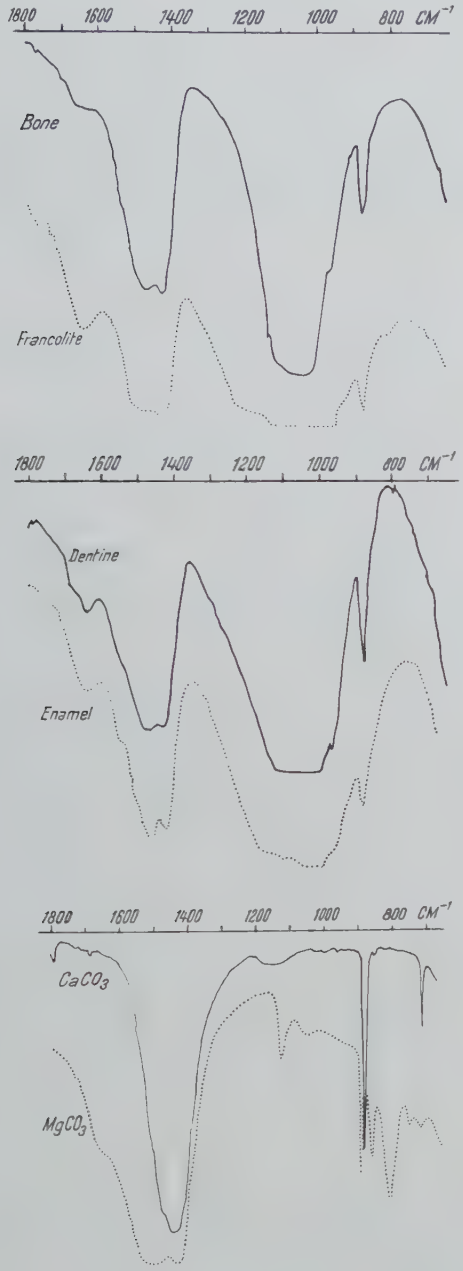

Fig. 2.–A comparison of the infra-red spectrum of dentine, enamel, bone and francolite with the standards calcite and magnesite. The large band appearing at about 1050 cm^{-1} is the orthophosphate band of apatite.

X-ray diffraction techniques alone have been unsuccessful in positively elucidating the character of the carbonate containing apatites. This is illustrated in Figure 1 which compares the X-ray patterns of the mineral francolite before and after about one third of the CO_2 has been leached out by lactic acid treatment. The two patterns are identical, showing the absence of the carbonate in the apatite phase, in a sort of negative manner.

Infra-red studies

It has been shown in the case of calcite that, as its particle size decreases, the intensity and resolution of the characteristic infra-red absorption spectrum increases[1]. This makes the infra-red absorption method ideal for characterizing a possible mixture of amorphous calcite and well crystallized apatite. Due to excessive broadening of the maxima, X-ray diffraction is unable to show crystals which are below about 100 Å in diameter. On the other hand, infra-red spectroscopy can demonstrate the presence of fine crystals.

The samples were prepared for examination by making a dry mixture of chemically pure KBr containing 2·5 % of the apatite. After mixing in a hand mortar, the mixture was pressed into a pastille of wafer shape (about 12 mm diameter and 1 mm thick) in a press which was constantly evacuated to avoid air and moisture entrapment and kept at a pressure of 10 tons for 5 min. The pastille was balanced against a standard pastille of pure KBr in a double beam Perkin-Elmer infra-red spectrometer and the spectrum taken between 1800 cm^{-1} and 650 cm^{-1}. The results are shown in Figure 2.

Conclusions

It seems apparent that there exists in francolite, enamel, bone and dentine a chemical bond between calcium and carbonate and between magnesium and carbonate which is identical to these bonds in calcite and magnesite. This would tend to support the negative X-ray diffraction evidence which shows no change in the apatite phase when the CO_2 is removed.

A. S. Posner and G. Duyckaerts

Institute of experimental Therapeutics and Laboratory of analytical Chemistry, University of Liège, March 23, 1954.

Résumé

La diffraction des rayons X et la spectrographie infrarouge ont été employées pour rechercher la présence de calcite et de magnésite dans la fraction minérale de l'os et des dents ainsi que dans le minéral qui lui est le plus proche au point de vue chimique, la francolite. Le résultat positif obtenu dans l'infra-rouge indique la présence de liaisons $CaCO_3$ et $MgCO_3$ associées à la matrice fondamentale du type apatite de ces substances.

[1] A. S. Posner and S. R. Stephenson, J. Amer. Dent. Assoc. *46*, 257 (1953).

[1] G. Duyckaerts and R. Lejeune, Spectrochimica Acta (in publication).

Multiple Spots on Paper Chromatograms

It is normally assumed that pure organic compounds give single spots on paper chromatograms and there is no doubt that, in the majority of cases, the assumption is justified. There are, however, various recorded instances of pure compounds giving multiple spots. In some cases the development of several spots is due to the formation of a new molecular species under the conditions of chromatography. Glucuronic acid gives spots corresponding to the original compound and the

cognate lactone, glucurone[1]. Sugars may give multiple spots[2] and there is evidence in one case that this is caused by the formation of new compounds under the influence of alkaline impurities in the paper[3]. The multiple spots obtained with lysine[4], using phenol-water, are attributed to the formation of association complexes with the solvent. Vitamin B_{12} may give spots corresponding to both Vitamin B_{12} and Vitamin B_{12b}[5]. In the case of phosphate esters it appears that ghost spots, which remain at the origin after the bulk of the ester is eluted in the normal way, are due to the presence of alkaline impurities in the paper[6]. A similar explanation applies in the case of salicylic and sulphosalicylic acids[7]. The ghost spots do not appear when paper washed with dilute hydrochloric acid is used. Certain antibiotics may give two zones in the presence of salts. The existence of tautomers was offered as a possible explanation in the case of streptomycin[8].

Fig. 1.

Fig. 2.

Fig. 1.–Normal chromatogram of monamycin (chloroform-water) showing ghost spot (R_F 0·00) and mobile spot (R_F 0·92).

Fig. 2.–Interrupted chromatogram showing third spot.

There are other cases, however, where it is not possible to account for the results by postulating the formation of new compounds. Smith has drawn attention to the possibility of false separations in paper chromatography similar to those obtained in partition chromatography on columns and indicates that this may occur in the paper chromatography of Vitamin B_{12}[9]. Double-fronting, which, under particular conditions may lead to the development of double spots has been observed with proteins[1]. One of the earlier publications on paper chromatography has indicated that ghost spots may be formed when mixtures of amino acids containing inorganic salts are chromatographed. The effect was attributed to water-logging of the paper at the origin[2].

We have observed another case which cannot be attributed in any simple way to the formation of new compounds. In the course of a study on the paper chromatography of seven cardiac glycosides[3] it was shown that when chloroform-water was used as solvent a ghost spot always remained at the origin. We have obtained a similar result with the antibiotic monamycin. In this case the very high degree of sensitivity of the biological procedure for locating the antibiotic[4] made possible a closer study of the phenomenon. Two spots (R_F values: 0·00, 0·54, and 0·00, 0·92) were obtained in experiments using benzene-water and chloroform-water respectively at 30°C. Toluene, xylene and n-butanol saturated with water also gave two spots. The ghost spots obtained in all these cases were eluted readily with methanol. The development of the ghost spot was not influenced by the moisture content of the paper or of the eluting solvents. After prolonged periods of equilibration before the solvent was allowed to run, the ghost spot remained but covered a larger area. The effect persisted after the paper had been thoroughly washed, chromatographically, with dilute hydrochloric acid, with an aqueous solution of the disodium salt of ethylene diamine tetra-acetic acid (Versene), and with an ethanolic solution of 8-hydroxyquinoline, to remove metallic ions. Two simple experiments using monamycin and the solvent chloroform-water excluded the possibility of the effect being due to trace impurities. When the solution obtained by running the mobile spot off the end of the paper was concentrated and applied again to a paper strip, the ghost spot appeared on the second chromatogram. In the second experiment a chromatogram was interrupted at an intermediate stage so that the mobile spot passed approximately half-way down the paper. When the solvent had evaporated from the paper the chromatography was resumed in the usual way. In this case, in addition to the normal spots (R_F 0·00, 0·92) (Fig. 1), there was a new distinct spot at a point corresponding to that occupied by the mobile spot at the time of interruption (Fig. 2)[5]. Similar results were obtained using the cardiac glycoside digitoxin with chloroform-water as solvent except that when prolonged equilibration was involved the Raymond reaction[6] was not sufficiently sensitive to detect the relatively diffuse ghost spot.

We have confirmed that two spots (R_F 0·00, 0·95) are produced when azobenzene is chromatographed with hexane. It appears likely that the process involved in the formation of ghost spots with azobenzene and with certain alkaloids[7] is similar to that which applies in the case of monamycin and cardiac glycosides.

The results we have obtained do not permit any decision concerning the mechanism of this effect. They may be most simply attributed to partial irreversible

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[5] We are grateful to Dr. G. Bras for the photographs, Figures 1, 2.
[6] W. D. Raymond, Analyst *64*, 113 (1939).
[7] J. B. Schute, *Mededelingen van de Vlaamse Chemische Vereniging 1*, 15 (1953).

adsorption of the solute on a cellulose phase[1] when the mobile solvent phase is absent.

[1] We are indebted to the Colonial Products Research Council for a grant in support of this work.

C. H. Hassall and K. E. Magnus

Chemistry Department, University College of the West Indies, Jamaica, December 12, 1953.

Zusammenfassung

Bei der papierchromatographischen Prüfung bestimmter reiner Stoffe wurde die Bildung mehrerer Flecken beobachtet. Derartige «ghost»-Flecken entstehen mit gewissen herzaktiven Glykosiden und auch mit dem Antibiotikum Monamycin.

[1] A. J. P. Martin, Ann. Rev. Biochem. *19*, 517 (1950).

Paper Electrophoretic Separation of Tuberculin Constituents

In the course of our investigations into the immune response of the host to tuberculous infection and antituberculosis vaccination, the problem arose as to how to determine the purity of the isolated antigenic fractions by a quick method and on a microscale. The recent successful application of zone electrophoresis on paper for the separation of serum proteins, enzymes, etc.[1] induced us to investigate this method for our purposes. Some of the results are presented in this preliminary note.

Two types of apparatus were used–one similar to that described by Kunkel and Tiselius[2] in which the paper is placed horizontally between two glass plates, and the other similar to the one used by Grassmann and Hannig[3] but with room for 6–8 horizontally placed paper strips (35 × 4 cm).

Of the different buffers used, phosphate buffer pH 8·3, ionic strength $\mu = 0{\cdot}1$ and veronal/veronal sodium buffer pH 8·6, ionic strength $\mu = 0{\cdot}11$, gave the best results, the latter yielding better separations in the Grassmann and Hannig apparatus. Citrate buffers of pH 5·5 and 6·3 afforded no good separations, and in addition some of the proteins tended to precipitate out from their solutions in these buffers, whereas 2–10 % solutions of most fractions could easily be dissolved in the alkaline buffers. Buffers of much higher pH values have not been tried since denaturation of tuberculo-proteins occurs under such conditions. A thick filter paper (Munktell 20/150) was used.

The experiments were run for 19 h at 200 V and 14–24 mA at + 4°C, applying 10–20 µl of 2–10 % solutions of the antigens. The proteins were identified by the bromphenol blue method according to Durrum[4], and for quantitative measurement eluted with 0·01 *n* NaOH and measured in the Beckman spectrophotometer at 595 mµ. Crystalline bovine albumin was chosen as standard substance. In a parallel run the polysaccharides were directly identified on paper after periodate oxidation with fuchsin sulphite[5], or eluted with water and determined with anthrone according to Morris[6].

[1] For a review, see A. Tiselius and P. Flodin, Adv. Protein Chem. *8*, 461 (1953).
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[5] E. Köiw and A. Grönwall, Scand. J. Clin. Lab. Invest. *4*, 244 (1952).
[6] D. L. Morris, Science *107*, 254 (1948).

The photographic reproduction in Figure 1 illustrates the separation of two fractions and a mixture of these into their protein components. The result suggests that there is one component which is common to both fractions.

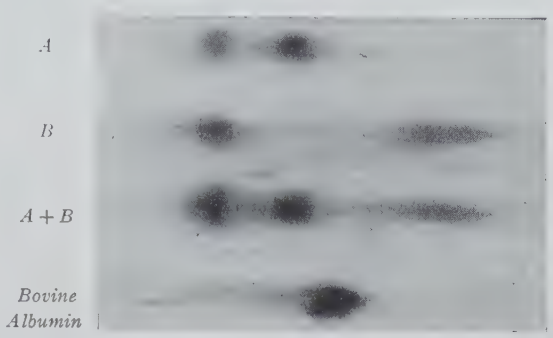

Fig. 1

Figure 2 indicates the usefulness of the method, in showing the heterogeneity of one particular fraction, thus confirming serological results which will be published shortly[1]. It is obvious that the polysaccharide part of the fraction moves rather slowly towards the anode, thereby affording a separation from proteins with higher mobilities. The curve for the proteins suggests the presence of three different components.

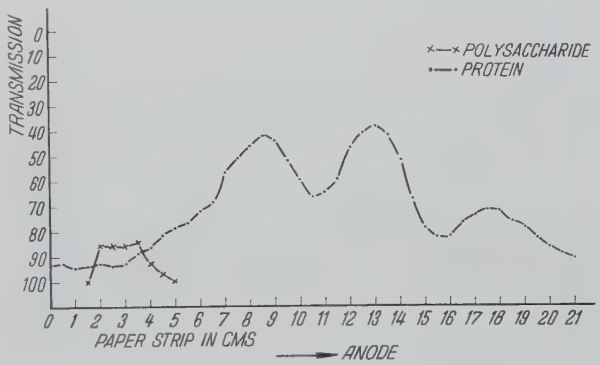

Fig. 2

There are, however, some antigens or antigenic mixtures which cannot yet be satisfactorily separated under these conditions. Heated fractions, e.g. PPD, are not separated into single spots, although they are known to contain at least two proteins with distinct serological specificities. Some unheated proteins, such as a highly purified protein corresponding to Seibert's C protein, although serologically nearly pure, show considerable tailing, probably due to strong absorption into the paper. They do not separate into single spots with the electrophoresis method as described above.

J. M. Rhodes and E. Sorkin

Tuberculosis Immunization Research Centre, c/o Statens Seruminstitut, Copenhagen, July 9, 1954.

Zusammenfassung

Methoden zur papierelektrophoretischen Trennung von antigenen Komponenten von Tuberkulin werden beschrieben.

[1] S. V. Boyden and E. Sorkin, To be published.

Wechselwirkungen zwischen fördernden und hemmenden Substanzen der Blutgerinnung

Es ist bekannt, dass eine Thrombokinase in Gegenwart einer zweiten anderer Herkunft Plasma in kürzerer Zeit zur Gerinnung bringen kann, als wenn sie allein in optimaler Menge verwendet wird[1]. Ähnliche Aktivitätssteigerungen können auch nach Zugabe von an sich inaktivem Serum[2] oder von verschiedenen, in der Literatur beschriebenen Faktoren[3] erzielt werden. Die dabei wirksamen Stoffe sind in ihrer chemischen Natur unbekannt. Die Mitteilung eines ähnlichen Phänomens mit chemisch teils gut definierten Substanzen, die physiologischerweise vorkommen und deren eine stark gerinnungsverzögernd wirkt, ist von besonderem Interesse.

An den zu beschreibenden Reaktionen, die mit Hühnerplasma als Gerinnungssubstrat ausgeführt wurden, nehmen u.a. der aus Schweinehirn hergestellte, lipoide, thermostabile Aktivator und der Aminoalkohol Sphingosin teil. Der genannte Aktivator beschleunigt die Gerinnung von Hühnerplasma und ist der ständige Begleiter der Kephaline, ohne jedoch mit dem eigentlichen Kephalin identisch zu sein[4]. Die zweite Substanz, Sphingosin, verzögert die Gerinnung stark; sie findet sich stets als mehr oder weniger starke Verunreinigung des Sphingomyelins. Diesem Umstand ist vermutlich die von Chargaff[5] beobachtete, gerinnungshemmende Wirkung der Sphingomyeline zuzuschreiben, da wir fanden, dass reine, sphingosinfreie Präparate keinerlei Einfluss auf die Gerinnung ausüben[6]. Die verwendeten Thrombokinasen sind in der Tabelle aufgeführt.

Wir beobachteten regelmässig, dass die Verkürzung der Gerinnungszeit des Plasmas durch den lipoiden Gerinnungsaktivator dann vergrössert ist, wenn das Gerinnungsmilieu gleichzeitig Sphingosin enthält. Gleiche Mengen Sphingosin allein verzögern dagegen die Gerinnung des Plasmas bis zu 24 h[7]. Wird das Plasma vor Zugabe des lipoiden Gerinnungsaktivators während einer Stunde mit Sphingosin bei 39°C inkubiert, so verkürzen sich die Gerinnungszeiten auf $^1/_3$–$^1/_6$ der ohne Inkubation erhaltenen Werte. Die 4. Spalte der Tabelle demonstriert dieses «Inkubationseffekt» genannte Phänomen. Aus den Werten der horizontalen, jeweils zusammengehörigen Reihen *a* und *b*, die innerhalb eines Versuches mit ein und demselben Plasma erhalten wurden, ist ersichtlich, dass der Einfluss selbst der doppelten Menge Sphingosin unter den gegebenen Versuchsbedingungen gering ist.

Nach Inkubation des lipoiden Aktivators mit Sphingosin ist die Aktivität dieser Mischung gegenüber Plasma unverändert. Es muss somit am Zustandekommen des Inkubationseffektes ein Bestandteil des Plasmas mitwirken[8].

Weitere Versuche zeigten, dass die beschleunigte Gerinnung in gleichem Ausmass auch durch direkte Zugabe bestimmter Thrombokinasen erzielt werden kann. Bei *gleichzeitigem* Zufügen von lipoidem Aktivator, Sphingosin und Thrombokinase aus Rinderlunge oder menschlicher Plazenta werden also *ohne* vorhergehende Inkubation des Plasmas mit Sphingosin Gerinnungszeiten erhalten (Spalte 6), die ebenso kurz sind wie diejenigen, die der lipoide Aktivator mit Plasma *nach* dessen einstündiger Inkubation mit Sphingosin ergibt (Spalte 4). Wie der Versuch *2b* zeigt, ist hierfür zuweilen die doppelte Sphingosinkonzentration erforderlich.

Der lipoide Aktivator, zusammen mit Thrombokinase aus Rinderlunge oder menschlicher Plazenta, gibt mit sphingosinhaltigem Plasma vor und nach Inkubation praktisch gleiche Gerinnungszeiten (Spalte 6).

Es handelt sich bei diesem Effekt nicht um eine Addition der Wirkungen von lipoidem Aktivator und Thrombokinasen. Die kurzen Gerinnungszeiten, wie sie mit dem lipoiden Aktivator, Thrombokinase und sphingosinhaltigem Plasma ohne Inkubation erzielt werden (Spalte 6), können so weder vom lipoiden Aktivator (Spalte 4) noch von den Thrombokinasen allein erreicht werden, von letzteren nicht einmal nach vorhergegangener Plasma-Sphingosin-Inkubation (Spalte 5). Das Entstehen einer zusätzlichen Aktivität scheint sicher zu sein.

In einer ausführlicheren Mitteilung werden wir zeigen, dass entsprechende Mischungen von lipoidem Aktivator und Thrombokinasen mit sphingosinfreiem Plasma nie die kurzen Gerinnungszeiten des sphingosinhaltigen Plasmas ergeben.

Die nach Quick aus Kaninchenhirn dargestellte Thrombokinase unterscheidet sich in ihrem Verhalten von den Thrombokinasen anderer Herkunft (Versuch 5 und 6). Dieser Befund steht im Einklang mit den ebenfalls abweichenden Ergebnissen bei unseren vergleichenden papierchromatographischen Studien über verschiedene Aktivatoren und mit deren Verhalten bei den klinischen Einstufen-Prothrombinbestimmungen. Ausserdem ist auffallend, dass mit Quickscher Thrombokinase und mit lipoidem Aktivator nach Inkubation des Plasmas mit Sphingosin prinzipiell eine starke Aktivitätssteigerung eintritt. Wir weisen in diesem Zusammenhang auf die Tatsache hin, dass beide Aktivatoren aus Hirngewebe bereitet werden.

Eine Diskussion der vorliegenden Befunde ist ebenso schwierig wie problematisch. Im menschlichen Blut kommen sowohl der lipoide Aktivator wie Sphingosin und, entsprechend dem Postulat der meisten Untersucher, eine Plasmathrombokinase vor. Falls letztere die gleichen Eigenschaften wie die von uns untersuchten Thrombokinasen aus Lungen- und Plazentagewebe zeigen sollte und alle Faktoren mit humanem Plasma und Hühnerplasma gleich reagieren, würden unserem Organismus alle für die oben beschriebenen Reaktionen notwendigen Stoffe zur Verfügung stehen. Ein Gleichgewicht dieser Faktoren würde eine normale Gerinnung gewährleisten, und Konzentrationsverschiebungen derselben könnten am Entstehen pathologischer Zustände mit beschleunigter (Thrombose) oder verzögerter Gerinnung (Hämophilie u.a.) mitwirken. Tocantins und Mitarbeiter[1] machen hierfür auf Grund langjähriger Untersuchungen eine Verminderung bzw. eine Erhöhung der Konzentration des von ihnen beschriebenen Inhibitors «Antithromboplastin» verantwortlich. Eigenen Untersuchungen zufolge halten wir den Inhibitor von Tocantins für identisch mit dem durch Sphingosin stark verunreinig-

[1] S. Gollub, F. E. Kaplan, D. R. Meranze und H. Tuft, Science *110*, 563 (1949). – S. Gollub, F. E. Kaplan und D. R. Meranze, Amer. J. Physiol. *162*, 293 (1950), und J. Lab. Clin. Med. *36*, 463 (1950).

[2] R. F. Jacox, J. Clin. Invest. *28*, 492 (1949).

[3] R. Biggs, Vortrag 4. Europ. Hämatologenkongress in Amsterdam, September 1953.

[4] A. Fischer und E. Hecht, Biochem. Z. *269*, 115 (1934). – E. Hecht, Sang *21*, 486 (1950).

[5] E. Chargaff, Science *85*, 548 (1937); J. biol. Chem. *121*, 175 und 187 (1937); *125*, 677 (1938).

[6] E. Hecht, Acta Haematol. *9*, 237 (1953).

[7] E. Hecht, Nature *167*, 279 und 633 (1951); Nederl. Tijdschr. Geneesk. *95*, 2371 (1951); Chem. Weekbl. *47*, 905 (1951).

[8] E. Hecht, Acta Haematol. *9*, 237 (1953).

[1] L. M. Tocantins und R. T. Carroll, *Blood Clotting and Allied Problems* (J. E. Flynn, New York 1949).

Sphingosineffekt und Thrombokinasen

Thrombokinasen aus	Nr.	Sphingosin in mm³	Gerinnungszeiten des sphingosinhaltigen Plasmas mit					
			lipoidem Aktivator		Thrombokinase		lipoidem Aktivator + Thrombokinase	
			vor Inkubation	nach Inkubation	vor Inkubation	nach Inkubation	vor Inkubation	nach Inkubation
Rinderlunge	1 a)	30	3′05″	42″	1′18″	1′20″	53″	1′06″
	b)	60	3′28″	1′02″	2′01″	1′53″	1′13″	1′04″
Rinderlunge	2 a)	30	3′35″	58″	1′30″	1′08″	1′50″	1′06″
	b)	60	3′23″	1′09″	1′33″	1′41″	58″	1′00″
Menschliche Plazenta	3 a)	30	5′23″	55″	2′30″	1′19″	53″	44″
	b)	60	4′55″	1′00″	2′41″	2′13″	50″	55″
Menschliche Plazenta	4 a)	30	9′35″	1′45″	3′15″	1′58″	1′45″	1′33″
	b)	60	9′30″	2′18″	4′00″	2′25″	1′43″	1′39″
Kaninchenhirn (Quick)	5 a)	30	4′00″	1′05″	10′55″	1′30″	6′30″	1′20″
	b)	60	4′25″	1′28″	13′00″	3′09″	5′00″	2′25″
Kaninchenhirn (Quick)	6 a)	30	3′54″	1′13″	21′00″	1′49″	6′49″	1′14″
	b)	60	4′23″	1′15″	17′50″	1′26″	6′18″	1′08″

Zusammensetzung des Gerinnungsmediums. 150 mm³ Hühnerplasma, 30 (a) bzw. 60 mm³ (b) Sphingosin (5 mg/1 cm³ H_2O) und 30 mm³ der jeweils angeführten Substanzen: lipoider Gerinnungsaktivator (6 mg/1 cm³ NaCl), Thrombokinase aus Rinderlunge (50 mg/1 cm³ NaCl), aus menschlicher Plazenta (Handelsprodukt der Amsterdamschen Chininfabrik «A.C.F.»-50 mg/1 cm³ NaCl) oder aus Kaninchenhirn nach Quick (20 mg/1 cm³ NaCl). Die Reaktionsmischungen wurden mit H_2O bzw. physiologischer NaCl-Lösung auf 270 mm³ gebracht. Technik der Gerinnungsbestimmungen nach Hecht[1].

ten lipoiden Aktivator[2]. Auf die Möglichkeit einer Verwendung unserer Befunde zur Stützung der von Tocantins 1954 neuerdings in erweiterter Form mitgeteilten Theorie der Blutgerinnung[3] kann an dieser Stelle nicht näher eingegangen werden.

Der lipoide Aktivator und Sphingosin könnten in geeigneten Mengenverhältnissen auch am Aufbau der «Plasmathrombokinase» beteiligt sein. Immerhin entwickelt einerseits der lipoide Aktivator mit sphingosininkubiertem Plasma eine Aktivität, die sonst nur den Thrombokinasen zukommt, und andererseits kann derselbe Effekt wie nach vorausgehender Plasma-Sphingosin-Inkubation durch gleichzeitige Zugabe von lipoidem Aktivator, Sphingosin *und* Thrombokinase zu Plasma auch ohne Inkubation erreicht werden. Zudem ist entsprechend der Auffassung von Howell[4] der lipoide, thermostabile Aktivator der wesentliche Bestandteil der meist thermolabilen Thrombokinasen.

Endlich erachten wir es für möglich, dass bei vielen in der Literatur beschriebenen gerinnungsfördernden und -hemmenden Faktoren die wirksamen Stoffe mit den Komponenten der von uns mitgeteilten Reaktionen identisch sind.

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Summary

The reactions of the lipoid activator and different thrombokinases, both alone and together with sphingosine-containing plasma, are described. In connection with the results reported, the possible significance of the physiological substances for the physiopathology of blood clotting, and the generation of plasma thrombokinase, is discussed.

1 E. Hecht, Acta med. Scand. *102*, 79 (1939).
2 E. Hecht, Acta Physiol. Pharmacol. Neerl. *2*, 134 (1951).
3 L. M. Tocantins, Blood *9*, 281 (1954).
4 W. H. Howell, Amer. J. Physiol. *31*, 1 (1912).

Ricerche farmacologiche sui lisati di organi

VII. Azione dei lisati di muscolo sul cuore di Rana bloccato mediante la prima legatura di Stannius

Premesse. Praticando una legatura a qualsiasi livello degli atri del cuore di Rana, a partire dal punto di sbocco del seno venoso nell'atrio destro, fino al limite del solco atrio-ventricolare, si provoca l'arresto immediato dell'organo in tutte le parti sottostanti la legatura, mentre quelle sovrastanti continuano a pulsare con ritmo inalterato (prima legatura di Stannius). Se nel cuore così bloccato si pratica una seconda legatura, in corrispondenza del solco atrio-ventricolare, il ventricolo ricomincia a pulsare (seconda legatura di Stannius). Il fenomeno s'ottiene sia sul cuore isolato che *in situ*.

Le classiche esperienze dello Stannius sono troppo note perché ci si debba soffermare a illustrarle in dettaglio; d'altra parte, tutte le ipotesi avanzate per poter interpretare l'intimo meccanismo dei fenomeni non hanno resistito ad una critica rigorosa[1]. Diremo soltanto che il blocco provocato dalla prima legatura è, generalmente, reversibile spontaneamente, per cui, dopo un periodo di arresto più o meno lungo, che può protrarsi fino a un'ora, le pulsazioni riprendono. Se, però, prima di operare la legatura, s'è provveduto a rimuovere ogni residuo di sangue, mediante un accurato lavaggio con liquido di Ringer, la riattivazione spontanea del cuore non s'ottiene, rimanendo esso inerte fino alla morte (Skramlik[2]).

Da ricerche che da tempo andiamo conducendo (Scarinci[3]; Scarinci e Zara[4]), sono emerse attività pecu-

1 Per una dettagliata rassegna sull'argomento, vedasi la monografia del C. J. Rothberger, in A. Bethe e G. Bergmann, *Handbuch der normalen und pathologischen Physiologie* (Julius Springer, Berlin 1926).
2 E. Skramlik, Pflüger's Arch. *183*, 120 (1920).
3 V. Scarinci, Arch. int. Pharmacodyn. *82*, 365 (1950).
4 V. Scarinci e E. Zara, Arch. Sci. biol. *37*, 388 (1953); *ibid.*, in stampa.

Fig. 1. Cuore isolato di Rana perfuso secondo Straub; alla freccia, s'inizia la perfusione con una soluzione di lisati di muscolo (2 p. 100) in liquido di Ringer (altre indicazioni nel testo).

liari dei lisati di muscolo sul cuore, specie se l'organo è reso insufficiente con artifici vari. L'attività cardiocinetica è stata messa in evidenza in diverse condizioni sperimentali, e l'effetto inotropo positivo rappresenta uno dei fenomeni più significativi e, quantitativamente, non raggiungibile con nessun altro farmaco, nemmeno con glucosidi ad azione digitalica (Fig. 1).

D'altra parte, ricerche di Küng[1] hanno dimostrato che un particolare estratto di cuore ha la proprietà di restaurare la capacità contrattile del cuore di Rana inattivo da diverse ore; risultati analoghi sono stati ottenuti da uno di noi con lisati di muscolo sul cuore in fibrillazione da affaticamento (Scarinci[1]). È da ricordare, infine, che le prime osservazioni che si riferiscono all'attività cardiocinetica degli estratti di cuore, sono dovute a Häusler[2].

Abbiamo, pertanto, voluto indagare l'azione dei lisati di muscolo[3] sul cuore di Rana arrestato mediante la prima legatura di Stannius; su tali ricerche riferiremo brevemente.

Risultati sperimentali e discussione. Le esperienze sono state sempre eseguite sul cuore isolato di Rana (*Rana esculenta*), sia immerso in un bagno ossigenato atto a conservarne la sopravvivenza, sia montato su una cannula secondo la tecnica di Straub. In ogni caso, la legatura veniva eseguita dopo circa un'ora dall'allestimento del preparato, con lo scopo di provvedere a un accurato lavaggio dell'organo.

I risultati sono stati positivi in ogni caso. Infatti, dopo la legatura, non appena il liquido di Ringer viene sostituito con la soluzione contenente i lisati (soluzione di concentrazione 2 p. 100), si ha la ripresa immediata dell'attività contrattile che appare subito ritmica, con pulsazioni più ampie di quelle basali (Fig. 2).

La funzionalità cardiaca si mantiene normale per lungo tempo; noi abbiamo mantenuto in efficienza cuori bloccati e immersi in un bagno ossigenato contenente lisati di muscolo, rinnovando il liquido ogni dodici ore, per tre giorni in ogni caso, e, alcune volte, fino a cinque giorni. A tale proposito, ricordiamo che il Küng[1], con osservazioni comparative dei tempi di sopravvivenza di cuori isolati di Rana, trattati con estratti di cuore e non trattati, ha dimostrato che i cuori trattati presentano un tempo di sopravvivenza notevolmente più lungo ed anche una capacità di lavoro più elevata, nei confronti dei cuori non trattati.

Nel nostro caso, se dopo aver ripristinato l'attività cardiaca con la soluzione contenente lisati, si lava con liquido di Ringer, si verifica di nuovo l'arresto del cuore, che può venire ulteriormente riattivato con la soluzione contenente lisati. Il fenomeno può essere ottenuto diverse volte sullo stesso preparato.

A nostro avviso, i punti più significativi dei risultati ottenuti nelle presenti ricerche sono due: 1. la ripresa immediata dell'attività cardiaca; 2. la ritmicità che perdura per lungo tempo.

Il primo fenomeno sottintende un principio avviatore; il secondo dimostra l'esistenza di fattori che consentono un'attività ritmica duratura.

Noi non conosciamo esattamente né la natura dell'arresto provocato dalla legatura, né le cause che determinano il ripristino spontaneo dell'attività automatica;

[1] H. L. Küng, Schweiz. med. Wschr. *80*, 205 (1950).
[2] H. Häusler, Arch. exp. Path. Pharmakol. *190*, 212 (1938).
[3] Esprimiamo i nostri ringraziamenti alla Ditta Braglia di Milano per averci gentilmente forniti i lisati da noi impiegati.

[1] H. L. Küng, Schweiz. med. Wschr. *80*, 205 (1950).

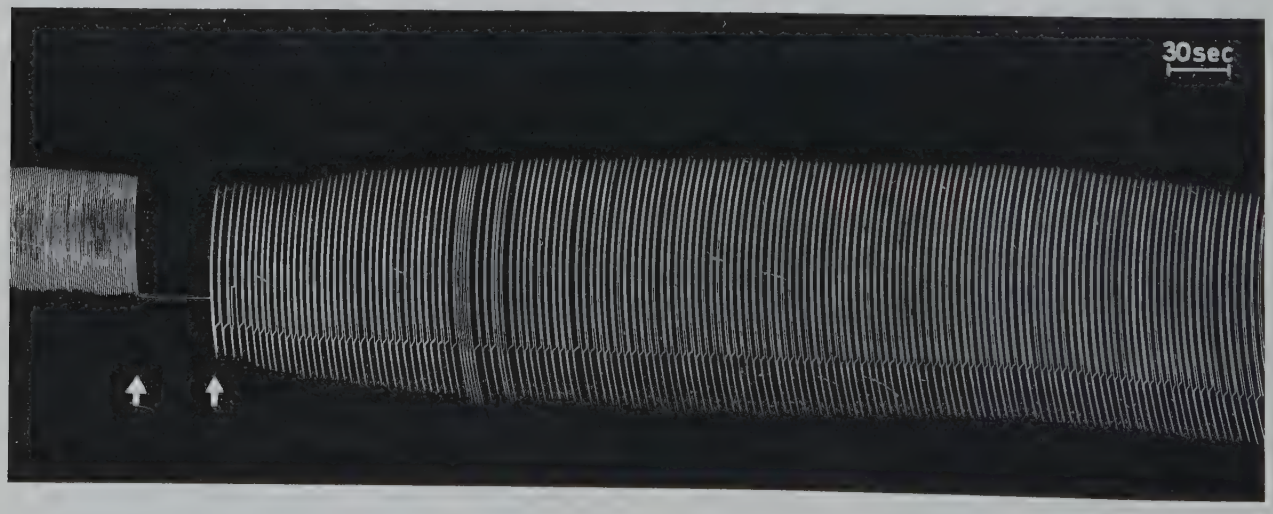


Fig. 2. Cuore isolato di Rana perfuso secondo Straub; alla prima freccia, viene operata la prima legatura di Stannius; alla seconda, s'inizia la perfusione con una soluzione di lisati di muscolo in Ringer (spiegazione e altre indicazioni nel testo).

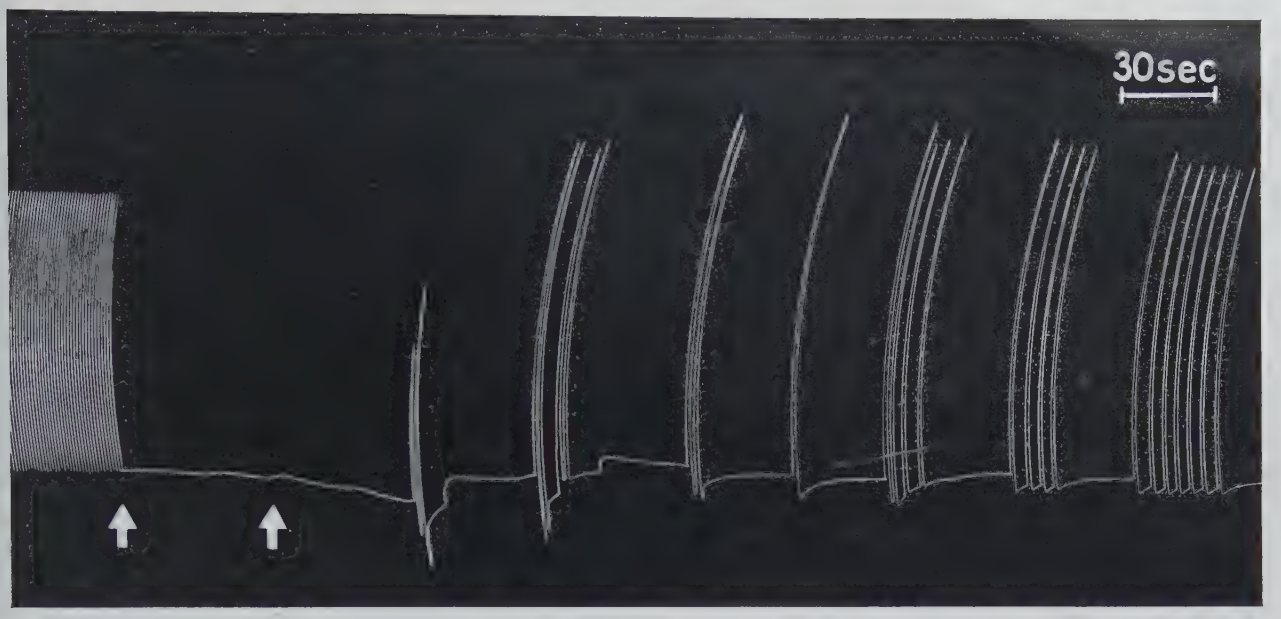


Fig. 3. Cuore isolato di Rana perfuso secondo STRAUB; alla prima freccia, viene operata la prima legatura di STANNIUS; alla seconda, s'inizia la perfusione con liquido di RINGER contenente una quantità di NaCl pari a 1/3 della normale, e reso isotonico con glucosio (vedasi la spiegazione nel testo).

ci sono note, tuttavia, alcune condizioni che abbreviano il tempo dell'arresto, come, per esempio, la composizione del liquido di perfusione. HOFFMANN[1] ha visto che se il cuore bloccato viene perfuso con una soluzione contenente poco cloruro di sodio e molto glucosio, s'ottiene il ripristino dell'attività cardiaca. In realtà, perfondendo l'organo arrestato con soluzioni ricche in glucosio, la contrattilità delle parti sottostanti la legatura viene riavviata; ma noi non siamo mai riusciti ad ottenere un'attività ritmica: in generale, si nota un ritmo periodico durante il quale le pulsazioni cardiache si succedono a gruppi, separati tra loro da pause più o meno lunghe (Fig. 3).

La durata del blocco dipende anche dalle condizioni di nutrizione del cuore (SKRAMLIK[2]).

I lisati di muscolo contengono diversi aminoacidi e sono particolarmente ricchi in istidina e arginina (0,260 g p. 100). Si poteva pensare che la riattivazione del cuore bloccato fosse in relazione con la presenza di tali corpi chimici; questo modo di vedere era avvalorato dai risultati di recentissime ricerche di MISLIN e RIESTERER[3] i quali hanno dimostrato, tra l'altro, che l'arginina, in soluzioni di concentrazioni variabili da 0,025 a 0,1 p. 1000, esercita un'attività regolatrice sulla funzione cardiaca. Noi abbiamo eseguito due ricerche collaterali: la prima, perfondendo il cuore arrestato con un liquido di RINGER cui erano stati aggiunti gli aminoacidi arginina e istidina in proporzioni tali da ottenere le medesime concentrazioni presenti in una soluzione di lisati al 2 p. 100; la seconda, perfondendo l'organo con le soluzioni di arginina adoperate da MISLIN e RIESTERER. I risultati sono stati negativi in ogni caso: infatti, non solo le soluzioni contenenti gli aminoacidi non hanno il potere di restaurare l'attività contrattile del cuore bloccato, ma, se dopo aver ottenuto la rianimazione dell'organo per mezzo di lisati, si perfonde con una qualsiasi delle soluzioni di aminoacidi suddette, il cuore si arresta nuovamente, per riprendere a funzionare quando viene ancora perfuso con lisati. In altri termini, nelle nostre condizioni sperimentali, le soluzioni contenenti aminoacidi si comportano come il liquido di RINGER normale.

È molto verosimile, tuttavia, che i lisati di muscolo contengano sostanze atte a migliorare il trofismo del miocardio; tale influenza eutrofica, però, sembra possa essere invocata solo in via subordinata, in quanto, se è vero che le condizioni di nutrizione possono accorciare il tempo di arresto, appare per lo meno poco probabile che tale influenza possa avere un effetto immediato. È presumibile che l'attività cardiocinetica dei lisati di muscolo entri in giuoco nel determinismo del fenomeno di riattivazione immediata della contrattilità.

Lo stimolo, infatti, di qualsiasi natura sia, meccanico, elettrico (corrente continua) o chimico, è capace di ridestare immediatamente l'attività contrattile del cuore bloccato dalla prima legatura di STANNIUS.

A proposito dello stimolo chimico, noi abbiamo visto, con altre ricerche collaterali, che soluzioni di ouabaina di concentrazione 1:20 000 possono riavviare immediatamente la contrattilità del cuore di Rana bloccato alla STANNIUS; però, in queste condizioni sperimentali, l'attività cardiaca si presenta disordinata e irregolare, osservandosi contrazioni molto rare e non ritmiche. Altrettanto dicasi per la stimolazione elettrica, in cui di preferenza si verificano serie di pulsazioni separate da intervalli più o meno lunghi. Tutto ciò dimostra che anche quando lo stimolo è costante e continuo, il cuore viene riavviato ma non s'ottiene mai un'attività regolare.

La conclusione che sembra possa trarsi dai risultati delle presenti ricerche è che i lisati di muscolo esplichino la loro azione con duplice meccanismo: cardiocinetico e eutrofico. Il primo sarebbe responsabile dell'immediata ripresa dell'attività del cuore, mentre il secondo entrerebbe in causa come regolatore di tale attività e condizionerebbe il perdurare di essa.

V. SCARINCI e E. ZARA

Istituto di Farmacologia e Farmacognosia, Università di Urbino, il 15 febbraio 1954.

[1] Citato da C. J. ROTHBERGER, in A. BETHE e G. BERGMANN, *Handbuch der normalen und Pathologischen Physiologie* (Julius Springer, Berlin 1926).

[2] E. SKRAMLIK, Pflüger's Arch. *184*, 21 (1920).

[3] H. MISLIN e L. RIESTERER, Helv. physiol. Acta *11*, C 67 (1953).

Zusammenfassung

Die Wirkung von Muskelextrakten auf das isolierte, durch die erste Stannius-Ligatur blockierte Froschherz wurde untersucht. Der Extrakt bewirkt die sofortige Wiederherstellung der Herztätigkeit, die sodann regelmässig längere Zeit anhält.

Die Wirkung der Muskelextrakte kann durch einen doppelten Mechanismus, cardiokinetisch und eutrophisch, erklärt werden.

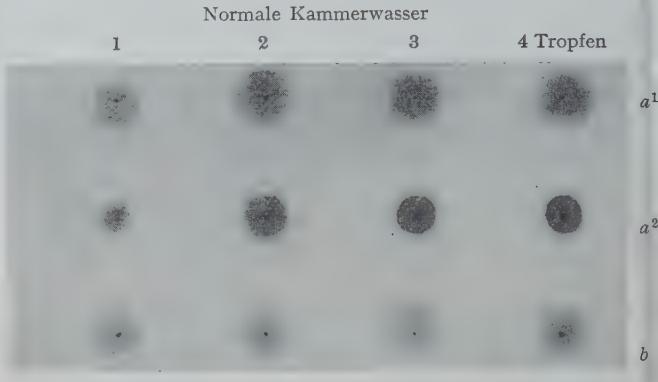
Neue mehrfache Untersuchungen am gleichen Kammerwasser menschlicher Augen

Eine einzelne Punktion von Kammerwasser beim Menschen erbringt höchstens ein Volumen von 0,25 cm³, meist jedoch ist nur mit 0,12 bis 0,18 cm³ zu rechnen. Beabsichtigt man jedoch, mehrere Untersuchungen am gleichen Kammerwasser durchzuführen, so müssen Mikromethoden herangezogen werden. Dazu haben wir folgenden Arbeitsgang ausgearbeitet: die sterile Ampulle, in welcher das Kammerwasser enthalten ist, wird geöffnet und ihr Gewicht plus Kammerwasser bestimmt. Anschliessend erfolgt die *Bestimmung des spezifischen Gewichtes* des Kammerwassers. Dazu werden erst Mischungen von Xylol und Brombenzol hergestellt, deren spezifische Gewichte so abgestuft sind, dass das zu erwartende spezifische Gewicht des Kammerwassers einem mittleren Wert gleichkommt. Für die unten im Abstufungszylinder befindliche Mischung verwenden wir 65,84 cm³ Chlorbenzol und 34,16 cm³ m-Xylol mit dem spezifischen Gewicht von 1,0181, darüber wird nach den Angaben von Vogt[1] die leichte Mischung vorsichtig aufgeschichtet. Sie enthält 60,85 cm³ Chlorbenzol und 39,15 cm³ m-Xylol mit dem spezifischen Gewicht von 0,9953. Als Standardlösungen richten wir uns drei Kochsalzlösungen von 0,43%, 0,86% und 1,29%, die das spezifische Gewicht 1,0021, 1,0045 und 1,0070 besitzen. Als Kapillarpipette verwenden wir die Aglapipette mit Mikrometervorschub, mit der Volumen von von 0,001 cm³ mit einer Genauigkeit von ± 0,00005 cm³ gemessen werden können. Das mittlere Tropfengewicht betrug bei unseren Untersuchungen 9,3 mg (doppelt destilliertes Wasser).

Für jede Messreihe wird zuerst mit den Standardlösungen der Dichtegradient der Abstufmischung geprüft. Die Ablesung am Präzisionszylinder von 250 cm³ Inhalt erstreckt sich im Mittel über 52 mm. Da eine Dichteschwankung von 0,5 mm gut abgelesen werden kann, sind Änderungen des spezifischen Gewichtes von 0,000047 noch messbar. Die stets durchgeführten Doppelbestimmungen benötigen 2 Tropfen aus der Aglapipette und lassen noch 16–20 Tropfen übrig, die zum Zählen der Zellen, zur Anlage einer bakteriologischen Kultur und zur *Bestimmung des Proteingehaltes* benutzt werden können. Dazu werden aus der gleichen Pipettenfüllung ein, zwei und drei Tropfen Kammerwasser auf einen Streifen Munktellpapier Nr. 150 (Grycksbo, Schweden) im Abstand von 3 cm aufgebracht.

Mit gleicher Technik werden auf demselben Papierstreifen Tropfen einer normalen, humanen Serumverdünnung von 10 mg% aufgebracht. Nachdem sie lufttrocken sind, werden die proteinhaltigen Stellen erst mit Amidoschwarz gefärbt (vgl. Wunderly)[2], darauf mit einem Stechbeutel von 15 mm innerem Durchmesser ausgestochen, fein zerschnitten und im Reagenzglas zur Elution mit 3 cm³ 50% Methanol, dem 5 g% Soda zugemischt ist, übergossen. Nach 8 h wird abgegossen, erneut mit 3 cm³ überschichtet und nach weiteren 8 h die Elutionsflüssigkeiten vereinigt und filtriert. Die kolorimetrische Auswertung erfolgt im Beckman D.U. auf Wellenlänge 595 mμ, wobei ein gleichgewonnener Leerwert des Papieres als Vergleichsflüssigkeit dient. Der Proteingehalt des Kammerwassers ergibt sich aus dem Vergleich mit den Extinktionen (Bezugswerten) der Serumverdünnung von 10 mg% Proteingehalt. Stets sind nur die Färbungen eines Papieres untereinander vergleichbar. Die Reproduktionsgenauigkeit liegt hier bei ± 3,7%.

Fig. 1.



a^1 erste Entnahme; a^2 zweite Entnahme, 30 min später; b Serumverdünnung von 10 mg% Proteingehalt.

Die Ampullen, aus denen das Kammerwasser in die Aglapipette gesogen wurde, werden erst mit NaCl phys. und dann mit H_2O dest. gewaschen und gewogen (Leergewicht). Die Differenz mit der ersten Wägung bringt das Gewicht der Kammerwasserabnahme (siehe Kolonne 1 in Tabelle I). Wenn die gefundene Menge Protein in mg% dazu in ein Verhältnis gesetzt wird, so erhält man die Angabe der Proteinkonzentration in γ. Schliesslich werden die *anorganischen Bestandteile* des Kammerwassers als Kochsalzionen in Rechnung gestellt, da das spezifische Gewicht an standardisierten Kochsalzlösungen vergleichend gemessen wurde.

Die untenstehende Tabelle gibt nun die Werte von 23 normalen Kammerwassern menschlicher Augen wieder, die überwiegend von amblyopen Augen mit einer Sehschärfe von unter $^3/_{60}$ und unter stets gleichbleibenden Bedingungen entnommen werden. Eine kritische klinische Würdigung bedarf eines weiter gespannten Rahmens und wird ebenso wie eine Zusammenstellung der Eiweissverhältnisse bei wiederholten Punktionen in Kürze folgen.

Um eine für die *Papierelektrophorese* hinreichend grosse Menge an Protein zu erhalten, ist es notwendig, zwei oder besser drei Kammerwasserpunktate in einer Ampulle zu vereinigen. Aus einer gläsernen Düse wird Pressluft eingeblasen, welche die Flüssigkeit rasch abdunstet. Wenn das Volumen etwa auf 0,02 cm³ zurückgegangen ist, wird alle Flüssigkeit in eine Kapillarpipette aufgesogen und alsbald punktförmig auf einen Munktell-Papierstreifen aufgetragen. Nach Papierelektrophorese werden die Spherogramme mit normalem Serumprotein verglichen, das gleich weit gewandert ist. Der Vergleich

[1] H. Vogt, Dissertation Zürich 1950.
[2] Ch. Wunderly, Chimia 7, 145 (1953).

Tabelle I

Nr.	Kammerwassergewicht 1	Spezifisches Gewicht 2	Proteingehalt 3	Proteingehalt 4	Anorganische Bestandteile in mg% 5
	mg		γ	mg%	mg%
2156	207,6	1,0056	20,5	9,8	950
2158	141,6	1,0060	28,1	19,3	1050
2167	142,5	1,0066	29,0	20,4	1150
2168	121,3	1,0056	22,3	18,4	950
2169	302,9	1,0068	18,1	27,0	1150
2171	110,1	1,0056	19,8	18,0	950
2200	185,4	1,0062	35,1	18,4	1100
2209	239,0	1,0055	21,0	8,8	1000
2210	189,7	1,0065	35,9	18,7	1150
2212	136,8	1,0059	30,1	22,3	1050
2213	267,8	1,0060	24,1	9,0	1050
2214	154,0	1,0060	30,1	20,1	1050
2215	278,3	1,0053	27,1	10,4	950
2218	115,0	1,0060	20,7	17,7	1050
2220	238,4	1,0066	30,9	13,3	1150
2221	216,0	1,0054	21,6	9,8	950
2225	257,2	1,0060	30,8	12,5	1050
2226	280,6	1,0061	26,9	9,8	1050
2145	159,0	1,0051	12,4	7,8	900
2231	219,5	1,0055	19,7	9,1	950
2203	164,7	1,0066	26,2	16,2	1150
2228	160,0	1,0063	19,2	11,7	1100
2216	141,1	1,0063	16,9	12,4	1100
23 Kammerwasser Mittel:		1,0059	24,6 γ	14,9 mg%	1043 mg%
Schwankung:		1,0068–1,0051	35,9–12,4	27,0–7,8	1150–900

gestattet ein Auftrennen nach einzelnen Fraktionen, die in sodaalkalischem 50%igem Methanol eluiert und deren Farbintensitäten im Beckman D.U. auf Wellenlänge 595 mμ bestimmt werden. Der geringe Proteingehalt lässt eine Unterscheidung von α1- und α2-Globulinen nicht zu, die Fehlergrenze der Bestimmung liegt bei ± 3,2% beim Albumin, bei ± 5,4% bei den Globulinfraktionen.

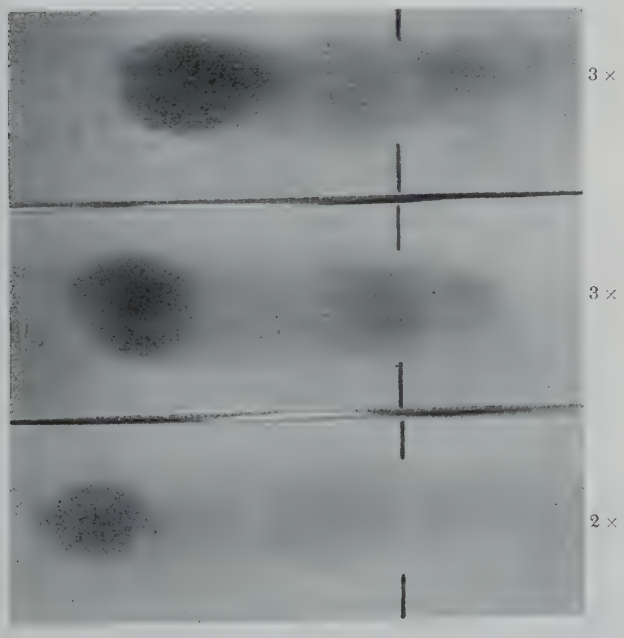


Fig. 2.
Normales Kammerwasser

Tabelle II

	Relative Prozente			
	Albumin	Globuline		
Elektrophoresen von		α	β	γ
2 Kammerwasserpunktaten	63	10	10	17
2 Kammerwasserpunktaten	61	10	12	17
3 Kammerwasserpunktaten	63	10	9	18
3 Kammerwasserpunktaten	66	7	10	17
3 Kammerwasserpunktaten	62	9	11	18
Mittelwerte	62,6	9,2	10,4	17,4
Serum, human, normal[1] . .	59,1	8,8	13,8	18,3
Liquor cerebrospinalis[1] (Lumbalpunktion) . . .	59,6	12,8	19,0	9,8

Gegenüber der Proteinzusammenstellung der von uns früher untersuchten krankheitshalber veränderten Kammerwasser besitzen die normalen durchschnittlich mehr Albumin und weniger α- sowie β-Globuline[2].

Ch. Wunderly, R. Steiger und H. R. Böhringer

Medizinische Klinik und Augenklinik der Universität Zürich, den 6. April 1954.

Summary

The normal human aequous has a volume of 0·12–0·20 cm³ and its protein content is from 10 to 20 mg%. We ascertained the specific weight by the falling drop method in a mixture of xylol and brombenzene. In order to estimate the protein content, we placed a series of drops from the human aequous on filter paper with the Agla pipette; they were then tinted with amido-black and the optical density of the eluted spots was read in the Beckman spectrophotometer. The remnants of 2–3 specimens of human aequous were then brought together and concentrated there until the protein concentration is just sufficient to allow a separation into the various fractions by paper-electrophoresis.

[1] G. Rossi und G. Schneider, Klin. Wschr. *31*, 969 (1953).
[2] Ch. Wunderly und B. Cagianut, Ann. Occul. *185*, 414 (1952).

Perfusion Studies Employing α-C^{14} Acetate to Study the Synergistic Effect of Cortisone and Insulin on Lipogenesis

This note reports preliminary observations on the synergistic effect of insulin and cortisone on fatty acid synthesis in the rat liver. A variety of observations on the effect of adrenal cortical hormones[1] and of insulin[2] on lipid metabolism have been reported, however, no information concerning the metabolic interplay of these two hormones has been demonstrated. The unequivocal demonstration that acetate acts as a precursor in the biosynthesis of fatty acids and cholesterol under *in vivo* and *in vitro* conditions[3] makes it possible to study these

[1] D. J. Ingle, J. Clin. Endocrinol. *10*, 312 (1950).
[2] D. R. Drury, Amer. J. Physiol. *131*, 536 (1940). – Dew. Stetten, Jr., and B. V. Klein, J. Biol. Chem. *159*, 593 (1946).
[3] K. Bloch and D. Rittenberg, J. Biol. Chem. *145*, 625 (1942). – D. Rittenberg and K. Bloch, J. Biol. Chem. *160*, 417 (1945). – K. Bloch, E. Borek, and D. Rittenberg, J. Biol. Chem. *162*, 441 (1946). – K. Bloch and W. Kramer, J. Biol. Chem. *173*, 811 (1948). – A. Pihl and K. Bloch, J. Biol. Chem. *183*, 431 (1950).

hormonal effects in a quantitative manner. Although liver slices are able to synthesize cholesterol at a fairly rapid rate[1], fatty acid synthesis in this isolated system is considerably slower than in the intact animal, and only small amounts of isotopic material are incorporated under these conditions[2]. To study lipogenesis in a model system (physiological favorable, and yet well controlled), four rat livers[3] from the Wistar strain were perfused for 6 h according to the method of Miller *et al.*[4] Approximately 200 ml of continually oxygenated heparinized blood which had been diluted with 0·9% saline (1 volume blood, 1/3 volume 0·9% saline), and which had a final red cell hematocrit of 28–33 volume percent was used as the perfusion fluid. In this system a blood flow of about 2 ml per gram wet weight of liver per minute with a pressure head of about 25 cm of blood was maintained throughout the experiment. To this perfusion fluid were added 250 mg sodium α-C^{14}-acetate[5] containing $7{\cdot}3 \times 10^6$ disintegrations per minute and 300 mg glucose, in an effort to keep the glucose level near 300 mg percent. In addition, 40 units of Squibb's crystalline zinc insulin (1 ml) and 10 mg of cortisone (Merck) were employed in the experiments indicated in Table I. After the perfusion the livers were lyophilized and the lipids extracted and fractionated by classical methods[6]. Saturated and unsaturated fatty acids from the phospholipid and triglyceride fractions were separated by means of the lead-salt method[7]. Cholesterol was isolated and analyzed as the digitonide. The data summarizing lipid synthesis in four perfusion experiments are presented in Table I.

From Table I, it may be seen that cortisone and insulin, when acting concomitantly, exert a marked effect on fatty acid synthesis while not affecting cholesterol synthesis. The effect is greatest in triglyceride fatty acid synthesis, but also manifests it self in phospholipid fatty acid synthesis. Insulin alone produces a moderate increase in isotope incorporation into fatty acids, thus confirming the reports of other workers using liver slices[1]. The effect of insulin alone differs from the effect of combined cortisone and insulin in a quantitative manner as regards fatty acid synthesis. Furthermore, insulin alone differs quantitatively from the combination cortisone-insulin with respect to glucose utilization, as shown in Table II. It cannot be decided at this time whether the insulin effect observed here and also in liver slices is due to the presence of residual amounts of adrenal cortical hormones in liver and blood rather than an effect referable to insulin *per se*. Since cortisone and insulin when acting simultaneously on this perfusion system affected only the utilization of acetate for fatty acid synthesis, these hormonal regulators might be assumed to influence the utilization of "two-carbon" fragments for fatty acid synthesis in a specific manner without affecting the rate of cholesterol synthesis. It is of interest to note that only in the combined cortisone-insulin experiment is fatty acid synthesis more rapid than cholesterol synthesis. The fact that fatty acid synthesis is more rapid than cholesterol synthesis *in vivo*[2] may be referable to the combined hormone action normally existent in the intact animal and strengthens the feeling that the isolated liver, when perfused with blood, approximates this normal behavior when supplemented with insulin and cortisone.

It should be pointed out that the figures in Table I relating C^{14} activity of the lipids to total C^{14} dose added to the perfusion fluid does not represent the total amount of isotope incorporated into lipids since an appreciable portion of the lipids synthesized are found in the circulating blood. Data regarding the isotope concentration of plasma lipids will be published in full at a later date. It is probable that the addition of extra glucose in these experiments leads to lower specific activities of fatty acids synthesized if one accepts the present view that glucose is converted to fatty acids via the "two-carbon" fragment.

Table II contains data relating the C^{14} activities of residual liver "protein" (after defatting) and carbon dioxide evolved. The findings concerning blood glucose utilization are also shown in this table. The C^{14} is uniformly incorporated into the liver "protein" fraction.

The increase in cholesterol C^{14} activity is two-fold when cortisone alone is added to the perfusing blood. This phenomenon is being examined more closely, par-

[1] K. Bloch, E. Borek, and D. Rittenberg, J. Biol. Chem. *162*, 441 (1946). – R. O. Brady and S. Gurin, J. Biol. Chem. *186*, 461 (1950).

[2] K. Bloch and W. Kramer, J. Biol. Chem. *173*, 811 (1948). – A. Pihl and K. Bloch, J. Biol. Chem. *183*, 431 (1950). – R. O. Brady and S. Gurin, J. Biol. Chem. *186*, 461 (1950).

[3] The rats used as blood and liver donors were fasted for 18 h prior to the experiment.

[4] L. L. Miller, C. G. Bly, M. L. Watson, and W. F. Bale, J. Exp. Med. *94*, 431 (1951).

[5] The author is indebted to the Isotopes Branch, Oak Ridge National Laboratories, for making available the α-C^{14}-acetate used in these experiments.

[6] L. L. Miller, C. G. Bly, M. L. Watson, and W. F. Bale, J. Exp. Med. *94*, 431 (1951). – G. Popjak and M. L. Beekmans, Biochem. J. *46*, 547 (1950).

[7] T. P. Hilditch, *The Chemical Constitution of Natural Fats* (John Wiley & Sons, Inc., New York, 1949), p. 468.

[1] K. Bloch and W. Kramer, J. Biol. Chem. *173*, 811 (1948). – R. O. Brady and S. Gurin, J. Biol. Chem. *186*, 461 (1950).

[2] D. Rittenberg and K. Bloch, J. Biol. Chem. *160*, 417 (1945).

Table I

Hormone additions to perfusing blood		Phospholipid fatty acids: Total fatty acids C^{14} activity*	Phospholipid fatty acids: (% total) (C^{14} dose)	Saturated fatty acids	Unsaturated fatty acids	Triglyceride fatty acids: Total fatty acids C^{14} activity*	Triglyceride fatty acids: (% total) (C^{14} dose)	Saturated fatty acids	Unsaturated fatty acids	Cholesterol: C^{14} activity*	Cholesterol
None	(I)	5·1	(0·1)	6·6	3·6	7·9	(0·1)	9·7	5·5	115	(0·4)
Insulin	(II)	21·5	(0·7)	39·4	8·3	16·9	(0·2)	42·2	8·3	123	(0·4)
Cortisone	(III)	7·0	(0·2)	8·0	3·4	11·3	(0·1)	13·0	6·9	233	(0·7)
Cortisone + Insulin	(IV)	40·0	(1·4)	95·3	36·1	138·5	(1·9)	344·0	118·5	118	(0·4)

* C^{14} activity $\times 10^4$ disintegrations per minute per millimole. In the case of the fatty acids, an average molecular weight of 270 has been assumed. (Mostly palmitic, others range 250–300.) The cholesterol activities are expressed in terms of cholesterol calculated from the digitonide.

Table II

Hormone additions to perfusing blood	$C^{14}O_2$ % of total dose	% of total C^{14} dose per gram residual liver "Protein"	Utilization of glucose* mg
None . . . . . (I)	8·7	1·1	− 79·0
Insulin . . . . . (II)	4·7	0·9	+ 88
			− 57
Cortisone . . . . . (III)	3·3	1·0	− 87
Insulin + Cortisone . (IV)	7·0	1·1	+125
			−231

* The values given represent the actual cumulated disappearance (shown as —) of glucose from the perfusing blood, corrected for withdrawal of samples during the perfusion. In experiments II and IV an increasing amount of reducing substance was found in the plasma during the first three hours (denoted by +). This increase was followed by a disappearance during the remaining hours of the experiment.

ticularly in the light of recent observations on hypercholesterolemia occuring during cortisone therapy[1].

W. Wendel[2]

Department of Chemistry, Western Illinois State College Macomb, Ill., June 24, 1954.

Zusammenfassung

Der vorliegende Aufsatz berichtet über Beobachtungen eines synergistischen Effektes von Insulin und Cortison bei der Fettsäuresynthese in der durchströmten Rattenleber.

Dabei wurde Natrium-α^{14}-C-acetat als Tracer verwendet und um Acetat bei der Biosynthese von Cholesterin und Fettsäuren aus dem Zweierbruchstück zu vertreten.

Nach Lyophilisation der Leber, Extraktion und Fraktionierung der Lipide wurde gefunden, dass ins Cholesterin der mit Cortison behandelten Leber mehr radioaktiver Kohlenstoff eingetreten war, bei der Behandlung mit Insulin dagegen mehr in die Fettsäuren und schliesslich in dieselben doppelt soviel als ins Cholesterin bei Behandlung mit Insulin und Cortison zusammen.

Der Anstieg nach Anwendung von Insulin allein dürfte auf Spuren von Nebennierenrindenhormonen zurückzuführen sein, welche noch in Leber und Blut vorhanden waren.

Demnach darf angenommen werden, dass Cortison und Insulin, wenn sie gleichzeitig zur Wirkung kommen, von grossem Einfluss auf die Verwendbarkeit der Zweierbruchstücke sind.

Der Anstieg der Cholesterinsynthese nach Behandlung mit Cortison ist durchaus bedeutungsvoll im Hinblick auf neuere Beobachtungen über Hypercholesterinämien, welche während Cortisontherapie auftreten.

[1] D. Adlersberg, L. Schaefer, and S. R. Drachman, J. Amer. Med. Ass. *144*, 909 (1950).

[2] Present address: Biophysics Section, Galesburg State Research Hospital, Galesburg, Ill., June 24, 1954.

Factors Involved in Drug-Produced Model Psychoses[1]

The use of fibrous wool protein as a model of the structural surface of receptors, possibly involved in the mechanism producing a model psychosis by drugs, was suggested by three lines of evidence.

First, the strong affinity (sorption) of certain basic compounds to keratins of low sulphur content as shown by the Gram-positiveness of epidermal[2] and nervous[3] tissue.

Second, the accumulating evidence indicating the similarity in behavior between keratins of high sulphur content (e.g. wool) and the protein component of certain cell membranes[1].

Third, some preliminary experiments confirming the role of wool protein as a useful model for simulating some of those receptors for which certain drugs appear to compete[2].

Based on these premises, the affinity for wool[3] of the following four basic compounds was determined at pH 5·2: mescaline hydrochloride, methedrine hydrochloride, lysergic acid monoethylamide (LAE), and lysergic acid diethylamide (LSD), the latter two in form of their methanoltartrates. These drugs when administered to humans in the approximate range of dosage: 500 mg, 100 mg, 1 mg, and 100 γ respectively, cause a model psychosis characterized by hallucinations and related phenomena of similar intensity and duration[4]. The method of determining the affinity for wool used in this study[5] gives only relative values, but these were found to be proportional to the absolute values obtained by another method[6].

The results are presented in the figure. The log. of dosage of each of the four compounds which causes a model psychosis of comparable intensity and duration is plotted against the amount of the compound sorbed by wool.

Affinity, i.e. average millimoles $\times 10^{-2}$ of drug sorbed by 1 gm of wool	Single dose of drug	log. of single dose
Mescaline 0	0·5 g	−0·3
Methedrine . . . . 0·6	0·1 g	−1·0
LAE . . . . . 1·1	1 mg	−3·0
LSD . . . . . 2·6	100 γ	−4·0

[1] Saskatchewan Commitee on Schizophrenia Research. Supported by the Department of National Health and Welfare, Ottawa.

[2] R. Fischer, Exper. *9*, 20 (1953).

[3] P. Bailey, Rev. Neurol. (French) *82*, 1 (1950).

[1] R. Fischer and P. Larose, Can. J. Med. Sci. *30*, 86 (1952); J. Bact. *64*, 435 (1952). – P. Larose and R. Fischer, Research (London) *5*, 419 (1952). – R. Fischer, Exper. *9*, 335 (1953). – P. Larose and R. Fischer, Science *117*, 449 (1953).

[2] R. Fischer, J. Ment. Sci. (in press).

[3] What is meant by wool (intact wool), see R. Fischer and P. Larose, Can. J. Med. Sci. *30*, 86 (1952).

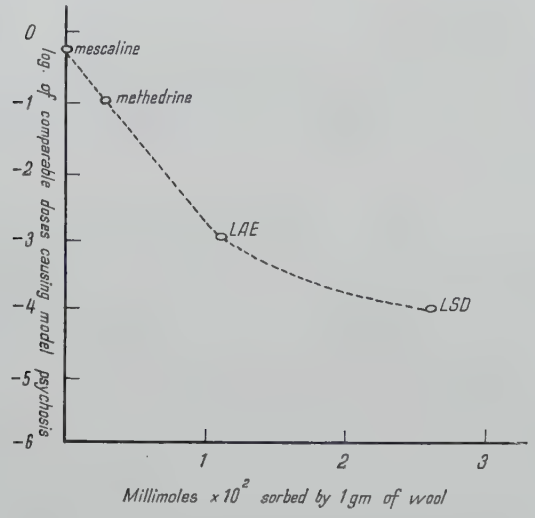
[4] K. Beringer, *Der Mezkalinrausch* (Springer-Verlag, Berlin, 1927). – W. A. Stoll, Schweiz. Arch. Neur. *60*, 1 (1947). – D. W. Liddell and H. Weil-Malherbe, J. Neurol. Neurosurg. Psychiat. *16*, 7 (1953). – W. Mayer-Gross, W. McAdam, and J. W. Walker, J. Mental Sci. *99*, 804 (1953); Nature (London) *168*, 827 (1951). – H. Solms, Schweiz. Med. Wschr. *83* 356 (1953). – R. Fischer, F. Georgi, and R. Weber, Schweiz. Med. Wschr. *81*, 817 (1951). – R. Fischer, Monthly Rev. Psychiat. Neurol. (Swiss) *126*, 315 (1953).

[5] R. Fischer, A. Hoelle, and S. Seidenberg, Helv. chim. Acta *34*, 210 (1951).

[6] P. Larose and R. Fischer, Schweiz. Z. Pathol. Bakt. *16*, 97 (1953).

The results indicate that the higher the affinity of a drug for wool, the lower the amount of that drug required to cause a model psychosis.

We are inclined to attribute the increasing affinity of the drugs in question for wool to their different degrees of specificity thus simulating a reversible inhibition of an equilibrium involved in the production of model psychoses.



We included only these four drugs in our Table and Figure because it is not easy to find other drugs which precipitate a reversible model psychosis with hallucinatory experience of comparable duration and intensity after administration of a *single* dose. Atabrine e.g., which is known to produce hallucinations and catatonic excitement, requires prolonged daily administration[1]. Its affinity was found to be 0·9 millimoles $\times 10^{-2}$/g of wool. Another antimalarial of similar structure and action, Pentaquine, displays an affinity of 3·6 millimoles $\times 10^{-2}$/g of wool. If Pentaquine is administered daily at a dosage four times higher than usual (0·12–0·24 g/day), it acts as an adrenergic blocking agent[2]. This feature seems to be, among others, a common characteristic of drugs capable of precipitating a model psychosis.

Liddell and Weil-Malherbe[3] have shown that methedrine as well as LSD seem to block adrenergic activity by lowering the blood-epinephrine level after short initial increase during the model psychosis; so might LAE since it belongs to the family of ergot alkaloids, which are presumed to inhibit sympathetic vasomotor tone[4]; however, it should be noted that the peripheral adrenolytic effects of LAE and LSD are about 300–2000 times weaker than that of dihydroergotamine[5].

Surgical (sympathectomy)[6] or chemical (Dibenamine)[7] adrenergic blockage, also produce psychotic experiences in certain subjects, possessing high epinephrine and norepinephrine[1] levels[2] to which they apparently are not adapted.

Hence it appears (a) that sympathetic stimulation followed by (peripheral) adrenergic blockage are factors involved in the production of model psychoses and (b) that drugs which exhibit their adrenergic blocking activity in smaller doses than e.g. Dibenamine and simultaneously display a high affinity for (wool) protein, are able to cause model psychosis in very small doses. Further aspects of the problem are to be considered in a more detailed report[3].

Acknowledgement. I am indebted to the psychiatric staff of the Saskatchewan (Mental) Hospitals in North Battleford, Weyburn and Regina, as well as to N. Agnew, Dr. A. Hoffer, Dr. S. Jedwab, Dr. H. Osmond, Dr. A. Szatmari, and A. Trew for stimulating discussions; to Dr. P. Larose, National Research Council, Ottawa, for valuable advice and criticism as well as for a generous gift of extracted wool; to the Sandoz Pharmaceuticals Ltd., Montreal, and especially to Professor E. Rothlin, Director, Sandoz AG., Basel (Switzerland), as well as to the Smith Kline & French, Laboratories, Philadelphia, for their kind assistance in providing LAE, LSD, and, SKF-501 as well as "pervitine" ("methedrine") respectively. Atabrine and Pentaquine were generously supplied by Winthrop-Stearns, Windsor, Ontario and Abbott Laboratories, Chicago.

R. Fischer

Research Laboratory, Munroe Wing, General Hospital, Regina, Saskatchewan, Canada, April 24, 1954.

Zusammenfassung

Mezkalin, Methedrine (Pervitin), Lysergsäuremonoäthylamid und Lysergsäurediäthylamid weisen eine steigende Affinität zu Wollprotein auf. – Es scheint, dass zwischen der Dosis, welche von diesen Substanzen benötigt wird, um beim gesunden Menschen nach einmaliger Verabreichung Modellpsychosen ungefähr vergleichbarer Intensität und Dauer hervorzurufen, einerseits und der Affinität derselben Substanzen zu Wollprotein andererseits eine umgekehrte Korrelation besteht.

[1] M. Nickerson, J. W. Henry, and G. H. Nomaguchi, J. Pharmacol. Exptl. Therap. *107*, 300 (1953).

[2] Only about 20% of hypertensive patients react to 0·5 g Dibenamine with a model psychosis.

[3] R. Fischer, J. Ment. Sci. (in press).

[4] S. W. Hoobler and A. S. Dontas, Pharmacol. Rev. *5*, 135 (1953).

[5] E. Rothlin, Private communication.

[6] G. Harrer and H. J. Urban, Nervenarzt *24*, 63 (1953).

[7] W. Walter-Buel, Monthly Rev. Psychiat. Neurol. (Swiss) *118*, 129 (1949).

The Differentiation of Optic Lobes Neurons in a Blind Cave Teleost

The structure of the most specialised neurons of the optic lobes of Ichthyopsida (except for Cyclostomata and Selachians) and Sauropsida is well known from the work of Ramon, Cajal, Van Geuchten, and others. They are mono- or bipolar spindle cells with one (sometimes two) "recurrent" branch emerging from the prolongment of the cell directed to the external surface of the lobe. These recurrent branches, considered as axons, after a narrow curve, run almost parallel to the cell down to the deepest layers of the lobe wall.

This very peculiar orientation of fibres was related by Leghissa (1946) to neurobiotactic antagonist effects created during development by the activation of two functional fields, a superficial one of optical nature and a deep one of general sensitivity (spino- and bulbotectal tracts).

It has also been demonstrated by many authors (Krause, Dürken, Larsell, and others, and, more recently, Filogamo (1948), Kollros (1947, 1948), Pflugfelder (1952) that the enucleation of one or both eyes determines an hypoplasia of one or both optic lobes. This phenomenon has been related, in Amphibians, to

the degeneration of nervous cells (Larsell) and also to a lower mytotic activity (Kollros) and, in the chicken, to fibre reduction and degeneration of cells (Filogamo). Hypoplasia was also observed in congenital anophtalmic animals.

The peculiarity of structure of these "recurrent neurons" brought me to a group of researches on the general problem of nervous differentiation. I refer the reader to some preliminary articles of mine and of my collaborators for some experimental results[1], while my purpose in this brief account is to describe the condition of these optic neurons in a blind Teleost from mexican caves, profiting by the experiment made by Nature itself by means of ecological adaptation.

The optic tectum of blind Teleosts was already taken into consideration by some authors [Ramsey (1901), Eigenmann (1909), Franz (1912), Charlton (1933)]: hypoplasia was observed with special reduction of the most superficial layers but was not considered from a cytological point of view.

I had the opportunity to dispose of numerous adult *Anophthyctys jordani* and of some embryos and larval stages of a spawn obtained in laboratory.

After the development of an optic cup almost of the size of zoologically related species living in the open air and with fully developed eyes, the organ enters into a rapid atrophy and reduces itself to a very small optic ball, with a very reduced cavity, formed by some retinic material, surrounded by a conspicuous pigmented tissue and a bone capsule, provided with a very reduced lens and an atrophyc optic nerve, very deeply located in the skull and completely covered by the integument (Fig. *B*).

Comparing the optic lobes of this blind fish with those of related species (*C*, *D*) with fully developed eyes (*A*) (*Astianax mexicanum*, *Pristella riddley*) of the same body size, hypoplasia is very evident; and the dorsal side and the *thorus semicircularis* are especially reduced; the optic superficial layer is absent and all the strata, but especially the second and third (following Huber and Crosby classification) are reduced in thickness. Spindle cells are decidedly reduced in number in agreement with the attribution of a specific optic function of these cells (Cajal, Kappers, and others). But the most impressive fact is the morphological aspect of these spindle-shaped optic cells: the form is typical and the recurrent branch is also present with its peculiar orientation (*F*), although there are no optic fibres.

These observations give a clear demonstration that the "recurrent axons" are not determined by conflicting physiological fields, as those suggested for vision and general sensitivity, acting neurobiotactically. They support the idea of an intrinsic nature of the specific "particular" neuron differentiation. Infact, the surviving optic neurons of the tectum, deprived of their optic function, differentiate normally, as also do the retinic cells of the remnant of the eye, without any possibility of physiological action.

Current experiments in my laboratory on the differentiation of these specific cells of the tectum clearly demonstrate that "recurrent neurons" differentiate also in small fragments of optic lobe of chicken implanted in the chorion-allantoic membrane at a very early stage of optic vesicle, apart from any physiological activity.

A. Stefanelli

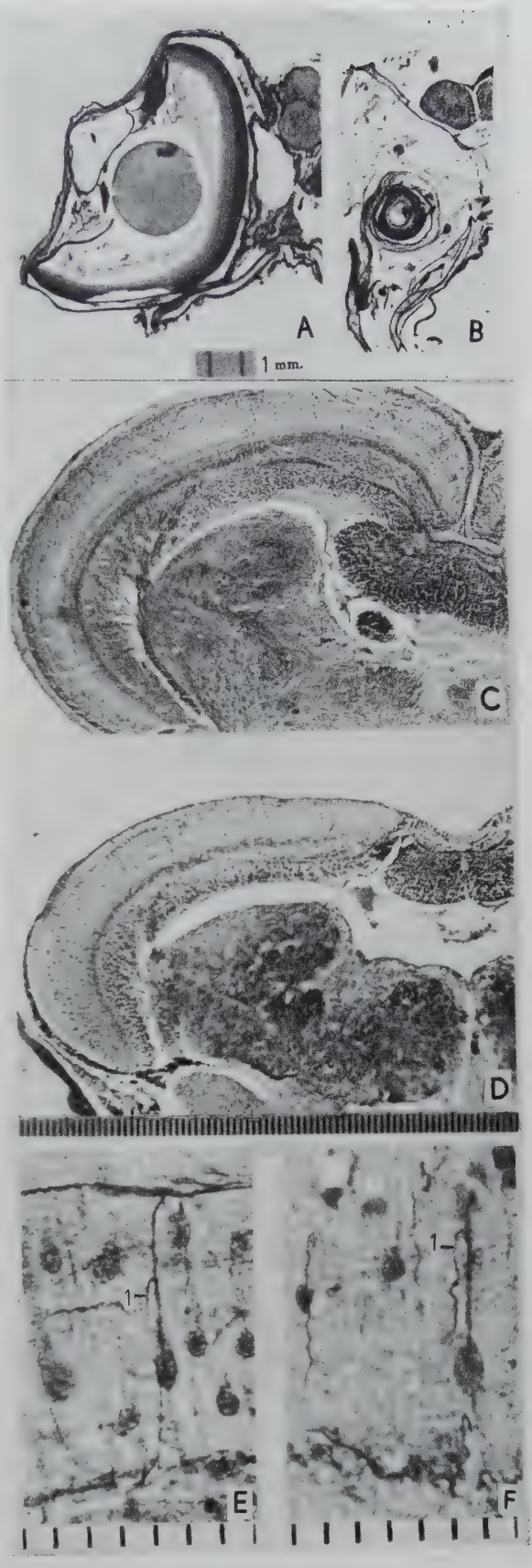


Fig. 1.–Right eye (*A*), optic lobe (*C*) and "recurrent neuron" (*E*) of *Astianax mexicanum* (open air living teleost); compared with those (*B*, *D*, *F*) of *Anophthyctys jordani* (cave fish zoologically related). Recurrent axon is indicated with 1 (*E*, *F*). Cajal-DeCastro method. Every space of the scale at the foot of pictures = 10 μ.

[1] A. Stefanelli, Rend. Acc. Naz. Lincei *16*, 277 (1954); *Fattore spaziale e organizzazione strutturale nel differenziamento dei neuroni dei lobi ottici e del cervelletto in impianti allanto-coriali nel pollo*, Rend. Acc. Naz. Lincei (in press); *Differenziamento ed evoluzione dei neuroni*, Scientia (in press). – A. Stefanelli and L. Chiti, Rend. Acc. Naz. Lincei *16*, 287, 291 (1954). – M. D'Ambrogi, Rend: Acc. Naz. Lincei *6*, 127 (1949).

Laboratory of Histology and Embryology, Faculty of Science, University of Rome, June 8, 1954.

Sommario

È comparato lo sviluppo degli occhi e del tetto ottico di un pesce cieco delle caverne (*Anophthyctys jordani*) con quello di forme affini viventi all'aperto e con occhi normalmente sviluppati. Pur essendovi una notevole ipoplasia del tetto i neuroni specifici a neurite ricorrente conservano le loro peculiari caratteristiche strutturali. Ciò dimostra che l'adattamento ecologico non ha ridotto i fattori intrinseci del differenziamento di questi neuroni.

PRO EXPERIMENTIS

An Experimental Method of Tissue Surface Measurements of Beta Activity *in vivo*

Several workers have considered the problem of *in vivo* external measurements of radioactivity in tissues. Studies have been carried out with β emitters and particularly with radiophosphorus by Low-Beer[1], Friedell *et al.*[2], Geffen *et al.*[3] on human subjects and by Sodaro and Sheppard[4], Schönenberg and Menzel[5] on rabbits. The physical aspect of these measurements has been investigated by Strajman[6] with theoretical and experimental analysis.

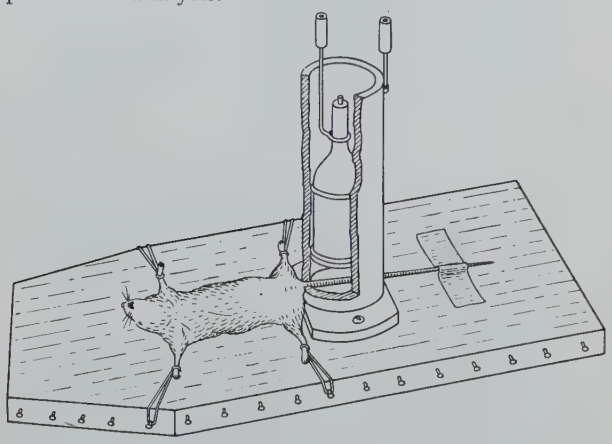


Fig. 1

As the interpretation of the results of external β activity measurements was not thorough, it seemed useful to study the behaviour of radiophosphorus in a normal animal by means of varying the route of administration only. We have therefore developed a simple and convenient experimental procedure, which enables us to obtain reliable surface β measurements in mice, over a lapse of some hours. Such procedure ensures strictly constant counting efficiency as well as rather comfortable conditions for the animal.

The mouse is laid on its back upon a small table with the four limbs held by means of rubber elastics. The tail is passed through two diametrically opposite small holes at the base of an aluminium cylinder firmly fixed on the table: the free extremity of the tail is then kept in place by adhesive tape (Fig. 1). Cotton wool, properly placed, prevents any spreading of radioactive excreta. The aluminium cylinder houses a β G.M. tube of the bell jar type with thin mica window. The thickness of the cylinder wall is sufficient to stop all β particles except those emitted from the counting area.

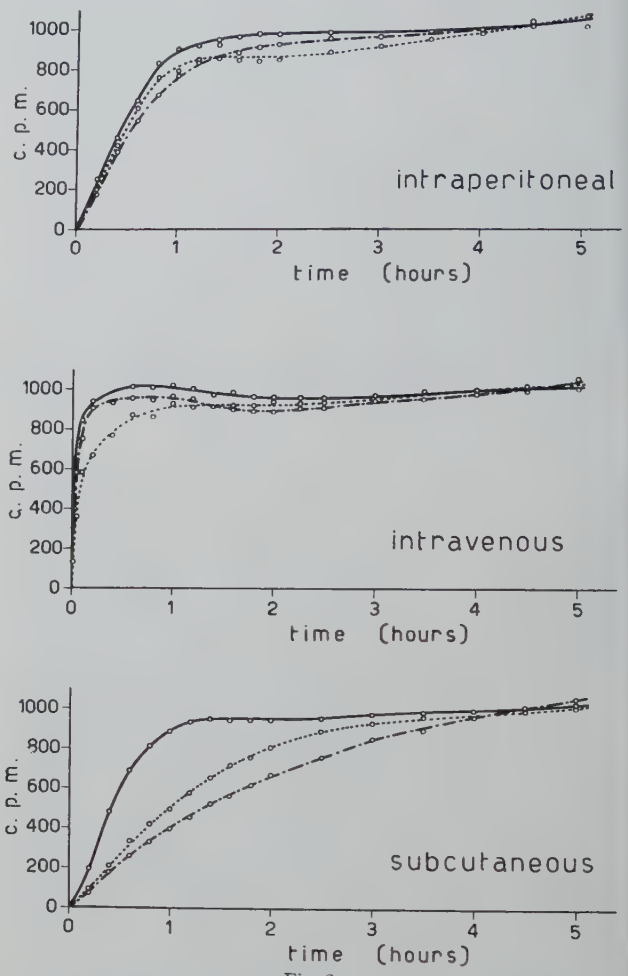


Fig. 2

We have employed white male mice of a body weight ranging from 23 to 27 g. The room temperature was constant (about 20°C). A solution of P^{32} in the form of Na_2HPO_4 with $\sim 85\ \gamma$ of stable phosphorus per ml of isotonic NaCl solution at pH = 7·2 was injected intraperitoneally (0·5 ml of solution), intravenously (0·1 ml into the vena jugularis) and subcutaneously (0·5 ml) respectively. We have made a series of brief integral preset-time countings for each animal starting before the injection and extended over 4 to 6 h.

Under these conditions of experiment, the counting rate depends on the following variables: (1) geometrical conditions of the counting (e.g. size of tail, portion of the tail in counting area, etc.); (2) route of administration; (3) quantity of injected P^{32}; (4) individual biological variability.

The counts-per-minute (corrected for dead time, background and radioactive decay) make "build up" curves when drawn in cartesian co-ordinates. A comparison between the "intravenous", "intraperitoneal" and "subcutaneous" curves indicates that their trend, different in the initial portions, tends gradually to become similar at the beginning of the third hour; thereafter the curves show a very slow and continuous slope of a few units per cent per hour. Furthermore the counting rate at the

[1] B. V. A. Low-Beer, *The Clinical Use of Radioactive Isotopes* (C. C. Thomas, Springfield, Ill., 1950).

[2] M. T. Friedell *et al.*, Arch. Int. Med. *83*, 608, 620 (1949); *85*, 667 (1950).

[3] A. Geffen, R. Loevinger, and B. S. Wolf, Radiology *46*, 856 (1951).

[4] R. M. Sodaro and C. W. Sheppard, Nucleonics *8*, No. 6, 40 (1951).

[5] H. Schönenberg and K. Menzel, Z. Kinderheilk. *73*, 17 (1953).

[6] E. Strajman, Univ. Calif. Publ. Physiology *8*, 333 (1951).

fifth hour after the injection is independent of the administration route. We registered 450 counts-per-minute per μc of injected P^{32} with a standard deviation of $\pm$ 33%. The "build up" curves can therefore be normalized by equalizing to 1000 the mean value of the counting-rate at the fifth hour. Figure 2 shows examples of the normalized curves.

The observation of these curves permits us to affirm that the geometrical and biological variabilities do not prevent the study of other variables introduced at will: one may study particularly the administration route (as we have done), the room temperature, the effects of the pharmacological substances administrated.

The discussion of the significance of our measurements is out of the scope of the present note. However, from the analysis (to be published) of these curves, and considering the results of our other researches, we can say that what is measured is the activity of the extracellular and cellular spaces, while the blood activity is not significantly measured.

T. Baglioni, C. Garavaglia, A. Perussia, e C. Polvani

Laboratori CISE; Istituto di Radiologia dell'Università; Divisione Biologica Sperimentale dell'Istituto Nazionale dei Tumori, Milano, January 26, 1954.

Riassunto

Gli autori descrivono una tecnica che consente di attuare determinazioni esterne di radioattività β su tessuti superficiali nel topo, mantenendo costante il rendimento di misura per una durata di alcune ore. Vengono presentati e brevemente commentati alcuni esempi di curve ottenute con questa tecnica in seguito ad iniezioni di P^{32} per via endovenosa, endoperitoneale e sottocutanea.

Informations - Informationen - Informazioni - Notes

THEORIA

An Electronic Theory of Transition Metal Enzymes and Catalysts

It is well-known that the transition metal is a good catalyst for oxidation and reduction. We have as industrial catalysts: platinum black, Raney's nickel, cobalt for hydrocarbon synthesis, and iron for ammonia synthesis; and as biocatalysts: heme-protein (cytochrome, cytochrome-oxidase, peroxidase, catalase, hemoglobin), and copper-protein (phenol- and ascorbic-acid-oxidase, hemocyanin).

In the heme-protein, an iron porphyrin and two histidines of protein sometimes form the d^2sp^3 octahedral six covalent iron complex salt[1]. As the 3d-orbitals of iron atom in the complex are saturated by the electrons of ligands, the 3d-electrons can be assumed to form a closed shell similar to the 3d-electrons of metal copper. It has been found that a bond radius of iron in the complex is about 1·23 Å[2], and an atomic radius of metal copper is about 1·27 Å[3]. The π-electrons of conjugated double bond in the iron porphyrin will be able to move freely among atoms just as do the 4s-electrons of metal copper. By taking it for granted that each of six nitrogen atoms of porphyrin and two histidines has the same relation to the central iron atoms and forms a symmetrical structure to the latter, an analogy to the metallic model that is, a polyhedron of metal copper calculated quantum-mechanically by Wigner-Seitz's method may exist as an isolated form in the prosthetic group of heme-protein. The copper-protein acts in the same way as does the heme-protein. Since the 3d-orbitals of copper atom are almost filled, the copper-protein will have no need to make a complex salt with the aid of porphyrin. The state containing 9 electrons in d-orbitals will be called hereafter "platinum-like state", since Pt is the most powerful catalyst for oxidation-reduction. One electron coming in and out of the platinum-like state may play a decisive role in the oxidation-reduction process for the metal enzymes, as shown in the Figure[4].

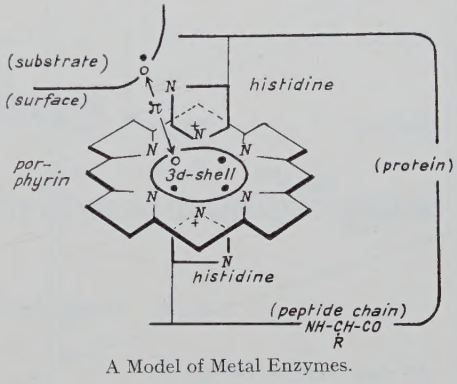
[1] C. D. Coryell and L. Pauling, J. Biol. Chem. *132*, 769 (1940). – H. Theorell and Å. Åkesson, J. Amer. Chem. Soc. *63*, 1812 (1941).

[2] L. Pauling, *The Nature of the Chemical Bond* (Cornell Press, 1939) p. 182; J. Amer. Chem. Soc. *69*, 542 (1947).

[3] F. E. Fowler, Smithsonian Physical Tables *1933*, 491.

[4] K. Ohmori and K. Ohki, Kagaku (Science) *18*, 36 (1948); Chem. Abstr. *45*, 1174 (1951). – K. Ohki, J. chem. Soc. Japan *70*, 68 (1949); C. A. *45*, 4135 (1951); Kagaku *19*, 152, 203 (1949); C. A. *45*, 2992 (1951); Kagaku *19*, 476 (1949); C. A. *46*, 3578 (1952); Kagaku *20*, 156 (1950); C. A. *44*, 6690 (1950). – H. Taketa and K. Ohki, Kagaku *20*, 186 (1950); C. A. *46*, 3398 (1952).– K. Ohki, *Quantum Chemistry in Biology* (Japanese edition, Kyoritsu Publ. Co., 1950).

Transition metal atoms are ordinarily arranged according to the well-known order of the periodic table by their physical and chemical properties. However, it has been found to be convenient for a survey of catalytic activities to rearrange them according to the number



A Model of Metal Enzymes.

of their d-electrons[1]. One of its representations is shown in Table where the number of d-electrons are taken from the Slater's treatise[2]. Hydrogenation by hydrogen molecule has taken place through the presence of 2nd class metals which include the 3rd class metals. Both Pt and Pd are the best catalysts for hydrogenation. Cu and Ni usually require some treatments in order to make these metals as much effective as catalysts as Pt, e.g. mixing CuO with Cr_2O_3 as in the case of Adkins' catalyst, or dissolving out Al by alkali from Al-Ni alloy to leave porous Ni as in the Raney's catalyst. In the d-electron deficient state, the metals will accept electrons from substrates and form at their surface some intermediate complex compounds with substrates which will later be decomposed and reduced, like Co in the Fischer's synthesis and Fe in the Haber's synthesis.

[1] K. Ohki, Kagaku *22*, 42 (1952); C. A. *46*, 2408 (1952); Kagaku *22*, 420 (1952); C. A. *46*, 9903 (1952); *Quantum Chemistry of Organic Compounds* (Japanese edition, Kyoritsu Publ. Co., 1952).

[2] J. C. Slater, *Introdution to Chemical Physics* (McGraw-Hill, p. 346, 347, 1939).

Table.–The Classification of Transition Metal Catalysts

No. of d-electrons	Transition metal catalyst: 6 period	5 period	4 period		Types of oxidation-reduction: addition reaction using 3rd class	hydrogenation by H_2: using 2nd class	hydrogenation by H_2: substrate	oxidation by O_2: using 1st class
10	Hg(10)	Cd(10)	Zn(10)	ox.				
10	Au(10) ?	Ag(10) ox.	Cu(10)	ox.	(copper-protein)	methanol syn. (hp.) Adkins' cat. (hp.)	C=O	formalin syn. copper-protein
		Pd(10)				Pd Pt } are the best cat.	C=C	
9	Pt(9)	(platinum-like state)						NH_3 oxidation sulfuric acid syn.
8	(2nd)	Rh(8)	Ni(8)		Reppe's reaction	Raney's Ni cat. fat hardening		use of natural gas
7	Ir(7)	(3rd) Ru(7)	Co(7)	ox.	Oxo-process (hp.) Co-complex salt	Fischer's syn.	C=O decomp.	drier of oil fatty acid syn.
6	(1st)							
	Os(6)ox.		Fe(6)	ox.	heme-protein	Haber's NH_3 syn. (hp.)	N≡N decomp.	NH_3 oxidation heme-protein
		Tc(6) ?				Bergius' method (hp.)	dest.	
5	Re(5)ox.		Mn(5)	ox.				
		Mo(5) ox. sulf.	Cr (5)	ox.				dehydrogenation
4	W (4) ox. sulf.							
3			V (3)	ox.				phthalic acid syn. sulfuric acid syn.

(1st class includes 2nd and 3rd classes, and 2nd class includes 3rd class)

(Abbreviations: ox.: oxide; sulf.: sulfide; syn.: synthesis; cat.: catalyst; hp.: high pressure; decomp.: decomposition; dest.: destructive hydrogenation).

Oxidation by oxygen molecule can be carried out in the presence of the 1st class metal oxides or sulfides which include the 2nd and the 3rd classes. The interesting reaction is the CO-addition where organic compounds having one more carbon atoms are synthetized. This reaction can be carried out by only the 3rd class metals, particularly, Ni, Co and their carbonyls, the example being one of Reppe's reaction and Oxo-process[1].

The substrates reduced by H_2 are mostly diatomic molecules like C=O, N≡N, O=O, furthermore, >C=C<, —C≡C—, —N=N—, C≡N, N—O, which consist of the 2nd period atoms and would be able to assume the triplet state of O_2. The oxidation-reduction of substrates would be carried out through the triplet state. S, Se, Te situated in the same column of the periodic table with O, as well as P, As with N, are strong inhibitors for the transition metal catalyst. The fact may be explained by assuming that they will behave in the same manner as O or N and compete with the substrates[1].

Consequently, it may be concluded that the oxidation-reduction process be most easily carried out by the closed shell structures having the large orbital angular momentum like the d-closed shell in atoms or the π-closed shell in molecules. In the well-known Lewis octet theory, he noticed the stability of the p-closed shell in atoms and the σ-closed shell in molecules. The present idea might be considered to be an extension of the Lewis' theory to the d- and π-closed shells.

Kosuke Ohki

Research Institute of Science and Industry, Kyushu University, Fukuoka, Japan, September 29, 1953.

Zusammenfassung

Zur Deutung der katalytischen Wirkung von Übergangsmetallen und Enzymen werden dieselben gemäss ihrem Elektronenaufbau der Aussenschalen klassifiziert, und es wird festgestellt, dass Oxydations-Reduktions-Prozesse über abgeschlossene *d*-Schalen in Atomen und abgeschlossene π-Schalen in Molekülen leicht stattfinden.

[1] Refer to the recent studies of catalysis as A. Couper and D. D. Eley, *Heterogeneous Catalysis*, Discuss. of Farad. Soc. *8*, 172 (1950). – D. A. Dowden and P. W. Reynolds, *Heterogeneous Catalysis*, Discuss. of Farad. Soc. *8*, 184 (1950). – O. Beeck, *Heterogeneous Catalysis*, Discuss. of Farad. Soc. *8*, 118 (1950). – G. M. Schwab, *Heterogeneous Catalysis*, Discuss. of Farad. *8*, 166 (1950).

[1] E. B. Maxted, Advances in Catalysis, Akad. Press *3*,129 (1951).

Die Erforschung der Wirkstoffe des Mutterkorns beschäftigte namhafte Chemiker und Apotheker während Jahrzehnten. Einen ersten Markstein bedeutete die Entdeckung und Reindarstellung des Alkaloids Ergotamin in den Laboratorien der Sandoz A.G., wo in der Folge auch die Auffindung und Herstellung weiterer für die Medizin äusserst wertvoller Mutterkorn-Reinalkaloide gelang. Neue therapeutische Aspekte eröffnete die erstmals bei Sandoz erfolgte Hydrierung dieser Stoffe. Folgende Präparate sind das Ergebnis dieser Forschungen:

Cafergot

Dihydroergotamin-Sandoz

Gynergen

Hydergin

Methergin

Neo-Gynergen

Partergin

SANDOZ

S A N D O Z A. G.

Unsere Neuerscheinungen

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